1997

Use of pyrimidine pathway to produce mutant plants that fail to respond to wound induction

Lan Zhou
Iowa State University

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Use of pyrimidine pathway to produce mutant plants that fail to respond to wound induction

by

Lan Zhou

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biochemistry

Major Professor: Robert W. Thornburg

Iowa State University

Ames, Iowa

1997

Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Lan Zhou

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Graduate College

DEDICATION

To my parents, Renjie Zhou and Shufang Zhang, for their love and understanding.

To my mentor, Dr. Robert W. Thornburg, for his guidance and encouragement.

TABLE OF CONTENTS

LIST OF NOMENCLATURE

ABA: abscisic acid

AP_5A: P^1, P^5-di(adenosine-5')-pentaphosphate

CAT assay: chloramphenicol acetyl transferase assay

CD: circular dichroism

CMP: cytosine monophosphate

codA: gene encoding cytosine deaminase

EMS: ethylmethane sulfonate

5FC: 5-fluorocytosine

5FOA: 5-fluoroorotic acid

5FU: 5-fluorouracil

GST: glutathione-S-transferase

HPLC: high performance liquid chromatography

IPTG: isopropyl-β-D-thiogalactoside

JA: jasmonic acid

k_{cat}.: catalytic constant

kDa: kilodalton

K_m: Michaelis constant

M1 plants: plants germinated from M1 seeds

M1 seeds: seeds after EMS treatment

M2 plants: plants germinated from M2 seeds

M2 seeds: seeds produced by M1 plants

M3 plants: plants germinated from M3 seeds

M3 seeds: seeds produced by M2 plants

MALDI: matrix-assisted laser desorption ionization

MS medium: Murashige and Skoog medium

NDP: nucleoside diphosphate

NMP: nucleoside monophosphate

NTP: nucleoside triphosphate

OMP: orotidine monophosphate

Ω: 5' untranslated tobacco mosaic virus leader sequence

PEI: polyethyleneimine

pGA482: a plant transformation binary vector

*pin*2: gene encoding proteinase inhibitor II

pNE3: a construct containing cauliflower mosaic virus 35S promoter, TMV Ω sequence and a bacterial *codA* gene

PRPP: phosphoribosyl pyrophosphate

pRT291: a construct containing *pin*2 promoter, a *Dictyostelium discoideum* UMP synthase gene and *pin*2 terminator, expresses UMP synthase in a wound-inducible manner in transgenic plants (Tr291)

pRT292: a construct containing cauliflower mosaic virus 35S promoter, a *Dictyostelium discoideum* UMP synthase gene and *pin*2 terminator, expresses UMP synthase constitutively in transgenic plants (Tr292)

pRT349: a construct containing *pin*2 promoter, a bacterial *codA* gene and *pin*2 terminator, expresses *codA* in a wound-inducible manner in transgenic plants (Tr349)

pRT354: a construct containing *Arabidopsis thaliana* nitrilase 1 promoter, a bacterial *codA* gene and *pin*2 terminator, expresses *codA* constitutively in transgenic plants (Tr354)

pRT380: a bacterial expression vector pGEX-4T-3 containing *Arabidopsis thaliana* UMP/CMP kinase cDNA

PVDF: polyvinylidene difluoride

*PYR*5-6: gene encoding UMP synthase

RPA: RNase protection assay

SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

T5 plants: the fifth generation of transgenic plants made by self-pollination

TLC: thin layer chromatography

TMV: tobacco mosaic virus

Tr25: *N. tabacum* transformed by the construct pRT45 containing *pin*2 promoter, a chloramphenicol acetyl transferase gene and *pin*2 terminator, expresses CAT in a wound-inducible manner

UMP: uridine monophosphate

UMP^{Dd}: *Dictyostelium discoideum* gene encoding UMP synthase

UMPS: UMP synthase

UP_5A: P^1-(5'-adenosyl) P^5-(5'-uridyl)-pentaphosphate

*ura*3 mutant: a *Saccharomyces cerevisiae* cell without OMP decarboxylase activity

*ura*5 mutant: a *Saccharomyces cerevisiae* cell without orotate phosphoribosyltransferase activity

*ura*6 mutant: a *Saccharomyces cerevisiae* cell without UMP kinase activity

ABSTRACT

To understand how proteinase inhibitor genes, as a part of plant defense system, are activated following wounding, we have developed a molecular genetic approach to directly select for mutants blocked in wound response in *Arabidopsis thaliana*. The wound-inducible promoter from the potato Proteinase Inhibitor II gene (*pin*2) was linked to a bacterial negative marker gene *codA* encoding for cytosine deaminase and transformed into *Arabidopsis thaliana*. The cytosine deaminase activity was detected in the leaves of the transgenic plants (Tr349) induced by sucrose, ABA, methyl jasmonate and mechanical wounding. Methyl jasmonate was the best for the *pin*2 induction in the seedlings among all the wound signal chemicals tested. Wild type seedlings had a dose-dependent sensitivity to 5FU. The seedlings were completely killed at a level of 100 μg/mL and 130 μg/mL 5FU for *Arabidopsis* and *N. tabacum*, respectively. 5FC at a concentration of 1 mg/mL had no effect on the seedlings of wild type of *Arabidopsis* or *N. tabacum*, or on *Arabidopsis* transgenic Tr349 seedlings without *pin*2 induction. However, 5FC had a dose-dependent effect on the seedlings of transgenic Tr349 with the *pin*2 induction by methyl jasmonate. No seedlings could survive above the concentration of 500 μg/mL 5FC. Homozygous Tr349 seeds were mutagenized by EMS. M2 seeds were collected and plated out on selective media containing 50 μg/mL kanamycin + 25 μM methyl jasmonate + 500 μg/mL 5FC. The 110 seedlings from M2 seeds survived on selective media. M3 seeds were collected and rescreened on selective media to eliminate false positives. Four M2 mutant lines that lost cytosine deaminase activity in M2 and M3 plants have been isolated.

Because of the long timeframes involved in the work with transgenic plants several smaller projects were also conducted. Due to the success of these side projects, they are included in this thesis as separate chapters. To test whether the UMP synthase gene from *D. discoideum* functions in plants or not, the construct *pin*2-UMPS^{Dd} was prepared and transformed to *N. tabacum*. The expression of the UMPS^{Dd} in the leaves of transgenic tobacco

was detected in a wound-inducible manner in both mRNA and enzyme activity. The UMP/CMP kinase cDNA from *Arabidopsis thaliana* was isolated, expressed in *E. coli* and characterized. Site-directed mutagenesis studies showed that a glycine-rich conserved sequence (P-loop) played a role in ATP-binding and enzyme catalysis.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Proteinase inhibitor genes

Proteinase inhibitor I was first discovered and crystallized from Russet Burbank potato tubers in 1962 (Ryan and Balls, 1962). Later proteinase inhibitor II was isolated from potato tubers (Bryant et al., 1976). Both inhibitors are serine proteinase inhibitors. They have evolved as two members of two nonhomologous gene families. Inhibitor I with a molecular mass of about 8000 KDa is an inhibitor of chymotrypsin. Inhibitor II with a molecular mass of 12,000 KDa is specific for chymotrypsin and trypsin. Initially, it was found that both inhibitors were synthesized in potatoes during tuber sprouting and development. However, the two inhibitors were found as transient proteins in leaves of potatoes and tomatoes (Ryan and Huisman, 1967; Ryan, 1968; Plunkett et al., 1982). In 1972, when Colorado potato beetles were applied to attack young tomato and potato plants, the plants responded to attack by synthesizing a large amount of the two inhibitors not only in the damaged leaves, but also in distal, undamaged leaves (Green and Ryan, 1972). Chymotrypsin-like serine proteases do not frequently exist in plants, but they are common in insect digestive tracts. Therefore, this was the first time that people realized the synthesis of the two inhibitors is wound-inducible and they are a part of plant defense system against insects. Since then, the research on understanding how proteinase inhibitors are synthesized following wounding has become an area of intense investigation.

Some wound signals that involve induction of the synthesis of proteinase inhibitors in plants have been identified. These wound signals include oligouronide fragments derived from plant cell walls (Bishop et al., 1984; Ryan, 1987), chitin and chitosan fragments from fungal cell walls (Walker-Simmons et al, 1983), an 18-amino-acid polypeptide from tomato leaves called systemin (Pearce et al., 1991), methyl jasmonate and jasmonic acid (Farmer and Ryan, 1990), abscisic acid (Peña-Cortés et al., 1989), auxin depletion (Kernan and Thornburg,

1989), sucrose (Johnson and Ryan, 1990), ethylene (O'Donnell et al., 1996; Yen et al., 1995), electrical signals (Wildon et al., 1992) and hydraulic pulses (Malone et al., 1994).

An octadecanoid signaling pathway for activation of proteinase inhibitor genes has been proposed (Farmer and Ryan, 1992a; 1992b). In this model, the pathway involves several intercellular and intracellular compartments. Systemic signals like systemin or localized signals such as oligogalacturonides and chitosan cause the activation or synthesis of lipases, which in turn, releases linolenic acid from either plasma or chloroplast membranes. Linolenic acid is then converted to phytodienoic acid (PDA) in the chloroplast. Subsequently jasmonic acid is synthesized from PDA through three β-oxidation steps probably in the peroxisomes. Jasmonic acid, the end product of the octadecanoid pathway, perhaps interacts with the receptors in nucleus and causes gene activation and subsequent accumulation of proteinase inhibitor proteins in the vacuole in the cytoplasm. This model is generally analogous to the eicosanoid pathway in animals, which was proposed to explain the accumulation of prostaglandins in response to polypeptide signals caused by stress (Samuelsson et al., 1978).

Some evidence has been found to support this lipid-based signal transduction cascade. Both jasmonic acid and its methyl ester have been found participating in the induction of defensive and secondary metabolites in at least 36 plant species (Mueller et al., 1993). Wounding caused a transient increase in endogenous jasmonic acid levels in leaves of tomato (Peña-Cortés et al., 1993; Doares et al., 1995b) and other plant species (Creelman et al., 1992; Albrecht et al., 1993). Levels of jasmonic acid were increased by systemin as wounding did in leaves (Doares et al., 1995a). Salicylic acid, an inhibitor of the octadecanoid pathway, strongly inhibited the accumulation of proteinase inhibitors by wounding, the accumulation of jasmonic acid (Peña-Cortés et al., 1993; Doares et al., 1995a) and the activation of defense gene by systemin (Doares et al., 1995a). A tomato mutant JL-5 with a defect in the octadecanoid pathway has been isolated (Lightner et al., 1993). The JL-5 mutant did not respond to wounding or to systemin, but responded to jasmonic acid. Also the finding that the

JL-5 mutant showed some localized induction of proteinase inhibitors suggested that both systemic and local signaling pathways occurred. Recent evidence showed that the JL-5 mutant had a defect between the synthesis of hydroperoxylinolenic acid and 12-oxo-phytodienoic acid (Howe et al., 1996). Research on how proteinase inhibitor genes are activated by wounding or other wound signals has provided us with incremental knowledge toward an understanding of the mystery of the signaling pathways leading to activation of proteinase inhibitor genes in response to wound signals. However, the mechanisms by which target cells perceive the signals and transduce them to ultimately activate proteinase inhibitor genes are not understood at all.

To understand the complex mechanisms involved in wound signal transduction pathways, we have developed a molecular genetic approach which permits us to directly select for mutants in the pathways. The strategy is based upon the selection of mutants blocked in activation of wound-inducible *pin*2 promoter.

Negative selection

In our mutant selection approach, we have chosen to use negative selection. A unique marker gene is expressed under the control of the wound-inducible *pin*2 promoter. The resulting construct is transferred to plant cells and mutagenesis is performed in the transgenic cells. Following the induction of *pin*2 promoter, cells expressing the marker gene in the presence of the non-toxic compound, will convert this into a toxic compound, and will die. Those cells which fail to express the negative selectable marker gene will survive the selection. This unique selection system is based on the observation that the metabolic product FdUMP inhibits thymidylate synthase (Kalpaxis et al., 1991). This results in a thymine starvation that kills cells (Chouini-Lalanne et al., 1989).

In our earliest selection experiments we used one marker gene UMPS. UMPS encodes UMP synthase, which is involved in *de novo* of pyrimidine metabolism (Figure 1). In some lower eukaryotes such as *Dictystelium discoideum* and in higher eukaryotes like mammals

(Traut and Jones, 1977), *Drosophila* (Rawls, 1978) and plants (Walther et al., 1984; Santoso, 1995; Maier et al., 1995), this single enzyme has both orotate phosphoribosyl transferase (OPRTase) and OMP decarboxylase (ODCase). UMP synthase genes or cDNAs have been isolated from some eukaryotes including *D. discoideum* (Boy-Marcott and Jacquet, 1982; Jacquet et al., 1988), *Arabidopsis thaliana* (Nasr et al., 1994) and *N. tabacum* (Maier et al., 1995). The non-toxic antimetabolite 5-fluoroorotic acid (5FOA) can be utilized by UMP synthase to form toxic 5-fluoro-UMP (FUMP). The cells can be rescued by uptaking uracil from the media through the salvage pathway. FOA selection has been successfully used in selecting mutants in eukaryotes including *Saccharomyces cerevisiae* (Boeke et al., 1984), *Candida albicans* (Magee et al., 1988), *Aspergillus flavus* (Woloshuk et al., 1989), *D. discoideum* (Kalpaxis et al., 1991), *N. tabacum* (Santoso and Thornburg, 1992) *and N. plumbaginifolia* (Santoso, 1995).

The other marker gene (*codA*) we used encodes cytosine deaminase. It catalyzes the conversion from cytosine to uracil in the salvage pathway (Figure 1). It also can catalyze the non-toxic substrate analog 5-fluorocytosine (5FC) to form toxic 5-fluorouracil. Most bacteria (Esders et al., 1985; Sakai et al., 1975a; 1975b; West et al., 1982) and many fungi (Hoeprich et al., 1974; Ipata et al., 1971) produce cytosine deaminase, but mammals and plants do not. Sensitivity of cells to 5FC has been studied in bacteria (Beck et al., 1972), yeast (Jund and Lacroute, 1970; Hoeprich et al., 1974), mammals (Hoeprich et al., 1974; Koechlin et al., 1966) and plants (Stougaard, 1993; Perera et al., 1993; Kobayashi et al., 1995). The *codA* gene as a negative selection marker has its unique advantage over other marker genes: plants do not have endogenous *codA* gene. This makes the selection simple without background cytosine deaminase activity.

At least three independent phenotypic classes of mutants have been isolated from *N. plumbaginifolia* (Santoso, 1995). One class of mutants is the knock-out mutant, in which only lost UMP synthase expression. We also have made a construct containing *pin*2 promoter

linked with *D. discoideum* UMP synthase gene (Shi and Thornburg, 1993). Before transformation of this construct into those UMP synthase knockout mutants to select the second-site mutations blocked in wound response, the construct was transformed to *N. tabacum* plants and the expression of *D. discoideum* UMP synthase gene was evaluated at different levels. However, difficulties with the growth and regeneration of the UMP synthase knockout mutants resulted in the abandonment of this strategy in favor of the cytosine deaminase strategy.

Dissertation Organization

This dissertation is presented as four journal papers preceded by a general introduction and followed by a general conclusion.

In Chapter 1, the background information about proteinase inhibitor genes and rationales of negative selections based on pyrimidine pathway for mutant selection are described. Chapter 2 forms the major part of dissertation. It presents the studies on the selection of mutants blocked in wound induction in *Arabidopsis thaliana* by the fluorocytosine selection scheme. Chapter 3 is devoted to evaluate the expression of construct *pin*2-UMPS^{Dd} at different levels in tobacco for the second-site mutation selection in wound response in *N. plumbaginifolia* by fluoroorotic acid selection. Chapter 4 and 5 focus on another key enzyme in *de novo* pathway of pyrimidine metabolism, UMP kinase from *Arabidopsis thaliana*. Chapter 4 presents molecular cloning, expression in *E. coli* and characterization. Chapter 5 includes further studies on UMP kinase by analyzing the conserved amino acid residues in the phosphate-binding site for ATP. In Chapter 6, a general conclusion is included.

References

Albrecht, T., Kehlen, A., Stahl, K., Knöfel, H.-D., Sembdner, G. and Weiler, E.W. (1993) Quantification of rapid, transient increases in jasmonic acid in wounded plants using a monoclonal antibody. Planta 191, 86-94.

Beck, C.F., Ingraham, J.L., Neuhard, J. and Thomassen, E. (1972) Metabolism of pyrimidines and pyrimidine nucleosides by *Salmonella typhimurium*. J. Bacteriol. 110, 218-219.

Bishop, P.D., Pearce, G., Bryant, J.E. and Ryan, C.A. (1984) Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves. J. Biol. Chem. 259, 13172-13177.

Boeke, J.D., Lacroute, F. and Fink, G.R. (1984) A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoroorotic acid resistance. Mol. Gen. Genet. 197, 345-346.

Boy-Marcott, E. and Jacquet, M. (1982) A *Dictyostelium* DNA fragments complements a *Saccharomyces cerevisiae ura*3 mutant. Gene 20, 433-440.

Bryant, J., Green, T., Gurusaddaiah, T. and Ryan, C.A. (1976) Proteinase inhibitor II from potatoes: Isolation and characterization of the isoinhibitor subunits. Biochem. 15, 3418-3423.

Chouini-Lalanne, N., Malet-Martino, C., Martino, R. and Michel, G. (1989) Study of the metabolism of fluorocytosine in *Aspergillus* by ^{19}K nuclear magnetic resonance spectroscopy. Antimicrobial-Agent and Chemotherapy 33, 1939-1945.

Creelman, R.A., Tierney, M.L. and Mullet, J.E. (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. USA 89, 4938-4941.

Doares, S.H., Narvaez-Vasquez, J., Conconi, A. and Ryan, C.A. (1995a) Salicylic acid inhibits synthesis of poteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol. 108, 1741-1746.

Doares, S.H., Syrovets, T., Weiler, E.W. and Ryan, C.A. (1995b) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc. Natl. Acad. Sci. USA 92, 4095-4098.

Esders, T.W. and Lynn, S.Y. (1985) Purification and properties of creatinine iminohydrolase *from Flavobacterium filamentosum*. J. Biol. Chem. 260, 3915-3922.

Farmer, E.E. and Ryan, C.A. (1992a) Octadecanoid precursors of jasmonic acid activate synthesis of wound-inducible proteinase inhibitors. Plant Cell 4, 129-134.

Farmer, E.E. and Ryan, C.A. (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc. Natl. Acad. Sci. USA 87, 7713-7716.

Farmer, E.E. and Ryan, C.A. (1992b) Octadecanoid-derived signals in plants. Trends Cell Biol. 2, 236-241.

Green, T.R. and Ryan, C.A. (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175, 776-777.

Hoeprich, P.D., Ingraham, J.L., Kleker, E. and Winship, M.J. (1974) Development of resistance to 5-fluorocytosine in *Candida parapsilosis* during therapy. J. Inf. Dis. 130, 112-118.

Howe, G.A., Lightner, J., Browse, J. and Ryan, C.A. (1996) An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. Plant Cell 8, 2067-2077.

Ipata, P.L., Marmocchi, F., Magni, G., Feliciolo, R. and Polidoro, G. (1971) Baker's yeast cytosine deaminase. Some enzymic properties and allosteric inhibition by nucleosides and nucleotides. Biochem. 10, 4270-4276.

Jacquet, M., Guilbaud, R. and Garreau, H. (1988) Sequence analysis of the *DdPYR*5-6 gene coding for UMP synthase in *Dictyostelium discoideum* and comparison with orotate phosphoribosyl transferases and OMP decarboxylases. Mol. Gen. Genet. 211, 441-445.

Johnson, R. and Ryan, C.A. (1990) Wound-inducible potato inhibitor II genes: Enhancement of expression by sucrose. Plant Mol. Biol. 14, 527-536.

Jund, R. and Lacroute, F. (1970) Genetic and physiological aspects of resistance to 5-fluoropyrimidines in *Saccharomyces cerevisiae*. J. Bacteriol. 102, 607-615.

Kalpaxis, D., Zundorf, I., Werner, H., Reindl, N., Boy-Marcotte, E., Jacquet, M. and Dingermann, T. (1991) Positive selection for *Dictyostelium discoideum* mutants lacking UMP synthase activity based on resistance to 5-fluoroorotic acid. Mol. Gen. Genet. 225, 492-500.

Kernan, A. and Thornburg, R.W. (1989) Auxin levels regulate the expression of a wound-inducible proteinase inhibitor II-chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. Plant Physiol. 91, 73-78.

Kobayashi, T., Hisajima, S., Stougaard, J. and Ichikawa, H. (1995) A conditional negative selection for *Arabidopsis* expressing a bacterial cytosine deaminase gene. Jpn. J. Genet. 70, 409-422.

Koechlin, B. A., Rubio, F., Palmer, S., Gabriel, T. and Duschinsky, R. (1966) The metabolism of 5-fluorocytosine-2^{14}C and cytosine-^{14}C in the rat and the disposition of 5-fluorocytosine-2 ^{14}C in man. Biochem. Pharmacol. 15, 435-446.

Lightner, J., Pearce, G., Ryan, C.A. and Browse, J. (1993) Isolation of signaling mutants of tomato (*Lycopersicon esculentum*). Mol. Gen. Genet. 241, 595-601.

Magee, P.T., Rikkerink, E.H.A. and Magee, B.B. (1988) Methods for the genetics and molecular biology of *Candida albicans*. Anal. Biochem. 175, 361-372.

Maier, T., Zhou, L. and Thornburg, R.W. (1995) Nucleotide sequence of a cDNA encoding UMP synthase from *Nicotiana tabacum*. Plant Physiol. 108, 1747.

Malone, M., Alarcon, J.J. and Palumbo, L. (1994) An hydraulic interpretation of rapid, long-distance wound-signaling in the tomato. Planta 193, 181-185.

Mueller, M.J., Brodschelm, W., Spannagl, E. and Zenk, M.H. (1993) Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc. Natl. Acad. Sci. USA 90, 7490-7494.

Nasr, F., Berthauche, N., Dufour, M.-E., Minet, M. and Lacroute, F. (1994) Heterospecific cloning of *Arabidopsis thaliana* cDNAs by direct complementation of pyrimidine auxotrophic mutants of *Saccharomyces cerevisiae*. I. Cloning and sequence analysis of two cDNAs catalyzing the second, fifth and sixth steps of the *de novo* pyrimidine biosynthesis pathway. Mol. Gen. Genet. 244, 23-32.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, D.J. (1996) Ethylene as a signal mediating the wound response of tomato plants. Science 274, 1914-1917.

Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 253, 895-898.

Peña-Cortés, H., Albrecht, T., Prat, S., Weiler, E.W. and Willmitzer, L. (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta 191, 123-128.

Peña-Cortés, H., Sanchez-Serrano, J., Rocha-Sosa, M. and Willmitzer, L. (1989) Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc. Natl. Acad. Sci. USA 86, 9851-9855.

Perera, R.J., Linard, C.G. and Singer, E.R. (1993) Cytosine deaminase as a negative selective marker for *Arabidopsis*. Plant Mol. Biol. 23, 793-799.

Plunkett, G., Senear, D.F., Zuroske, G. and Ryan, C.A. (1982) Proteinase inhibitor I and II from leaves of wounded tomato plants: purification and properties. Arch. Biochem. Biophys. 213, 463-672.

Rawls, J.M. (1978) The enzymes for *de novo* pyrimidine biosynthesis in *Drosophila melanogaster*: Their localization, properties and expression during development. Biochem. Physiol. 628, 207-216.

Ryan, C.A. (1968) An inducible protein in tomato and potato leaflets. Plant Physiol. 43, 1880.

Ryan, C.A. (1987) Oligosaccharide signaling in plants. Annu. Rev. Cell Biol. 3, 295-317.

Ryan, C.A. and Balls, A.K. (1962) An inhibitor of chymotrypsin from *Solanum tuberosum* and its behavior toward trypsin. Proc. Natl. Acad. Sci. USA, 48, 1839-1844.

Ryan, C.A. and Huisman, O.C. (1967) Chymotrypsin inhibitor I from potatoes: A transient component in leaves of young potato plants. Nature 214, 1047.

Sakai, T., Yu, T., Tabe, H., Omata, S.(1975a) Purification of cytosine deaminase from *Serratia marcescens*. Agric. Biol. Chem. 39, 1623-1629.

Sakai, T., Yu, T., Taniguchi, K. and Omata, S. (1975b) Purification of cytosine deaminase *from Pseudomonas aureofaciens*. Agric. Biol. Chem. 39, 2015-2020.

Samuelsson, B., Goldyne, M., Granström, E., Hamberg, M., Hammarström, S. and Malmsten, C. (1978) Prostaglandins and thromboxanes. Annu. Rev. Biochem. 47, 997-1029.

Santoso, D. (1995) 5-fluoroorotic acid-selected cell lines and regulation of UMP synthase gene expression in tobacco cells. Ph.D. thesis. Iowa State University, Ames.

Shi, N.Q. and Thornburg, R.W. (1993) Construction of a UMP synthase expression cassette from Dictyostelium discoideum. Gene 127, 199-202.

Stougaard J (1993) Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene. Plant J. 3, 755-761.

Traut, T.W. and Jones, M.E. (1977) Inhibitors of orotate phosphoribosyl-transferase and orotidine-5'-phosphate decarboxylase from mouse ehrlich ascites cells: a procedure for analyzing the inhibition of a multi-enzyme complex. Biochem. Pharmacol. 26, 2281-2291.

Walker-Simmons, M., Hadwiger, L. and Ryan, C.A. (1983) Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves. Biochem. Biophys. Res. Comm 110, 194-199.

Walther, R., Wald, K., Glund, K. and Tewes, A. (1984) Evidence that a single polypeptide catalyzes the two step conversion of orotate to UMP in cells from a tomato suspension culture. J. Plant Physiol. 116, 301-311.

West, T.P., Shanley, M.S. and O'Donovan, G.A. (1982) Purification and some properties of cytosine deaminase from *Salmonella typhimurium*. Biochim. Biophys. Acta. 719, 251-258.

Wildon, D.C., Thain, J.F., Minchin, P.E.H., Gubb, I.R., Reilly, A.J., Skipper, Y.D., Doherty, H.M., O'Donnell, P.J. and Bowles, D.J. (1992) Electrical signaling and systemic proteinase inhibitor induction in the wounded plant. Nature 360, 62-65.

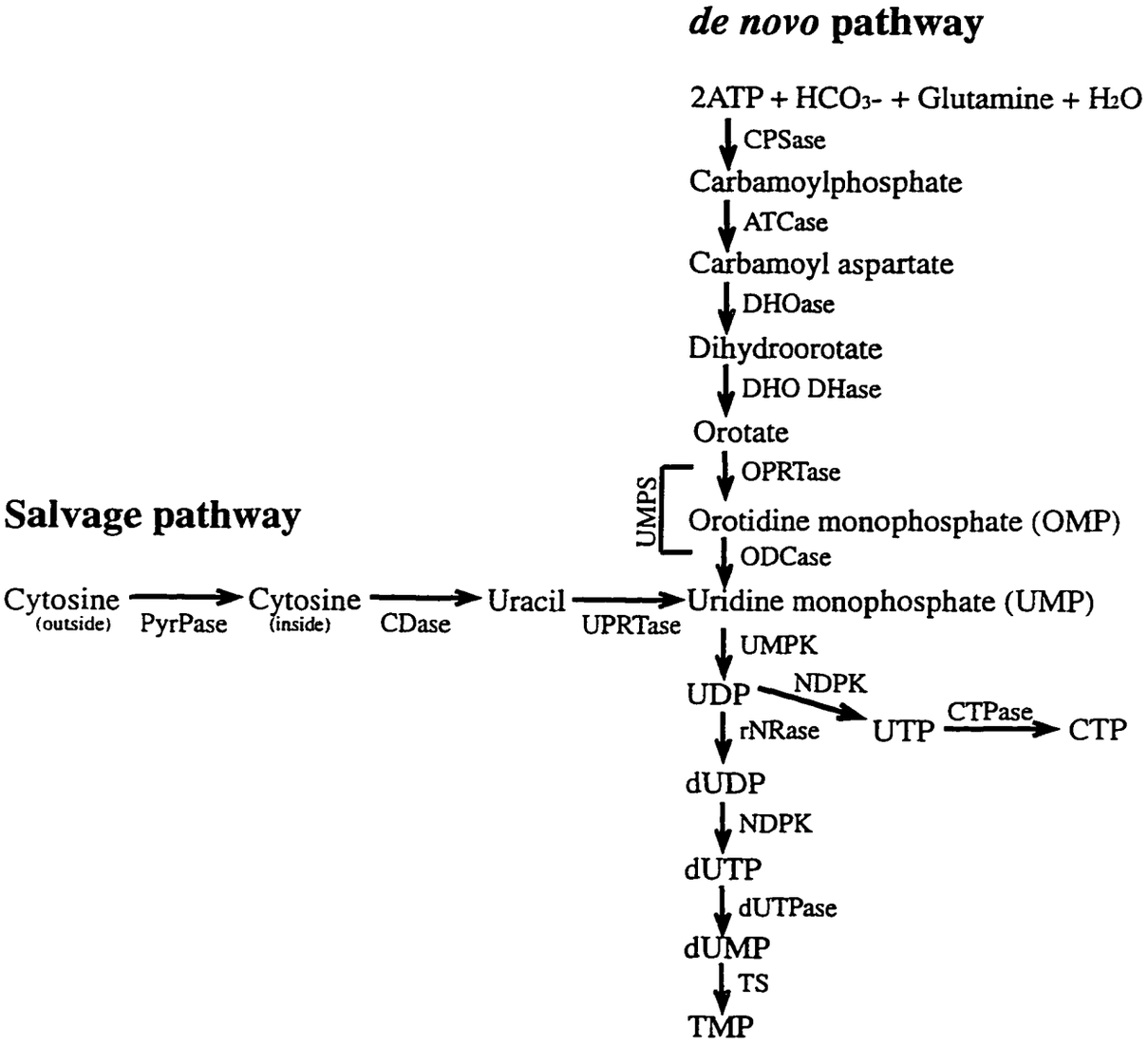
Woloshuk, C.P., Seip, E.R., Payne, G.A. and Adkins, C.R. (1989) Genetic transformation system for aflatoxin-producing fungus, *Aspergillus flavus*. Appl. Environ. Micro. 55, 86-90.

Yen, H.C., Lee, S., Tanksley, S.D., Lanahan, M.B., Klee, H.J. and Giovannoni, J.J. (1995) The tomato *Never-ripe* locus regulates ethylene-inducible gene expression and is linked to a homolog of the Arabidopsis *ETR*1 gene. plant Physiol. 107, 1343-1353.

Figure 1. Pyrimidine pathway

Enzymes involved in the pathway are CPSase = carbamoylphosphate synthase, ATCase = aspartate transcarbamoylase, DHOase = dihydroorotase, DHO DHase = dihydroorotate dehydrogenase, OPRTase = orotate phosphoribosyltransferase, ODCase = OMP decarboxylase, UMPK = UMP kinase, rNRase = ribonuleotide reductase, NDPK = NDP kinase, CTPase = cytidine 5'-triphosphate synthase, dUTPase = deoxyuracil triphosphotase, TS = thymidylate synthase, PyrPase = pyrimidine permease, CDase = cytosine deaminase, UPRTase = uracil phosphoribosyltransferase and UMPS = UMP synthase.

Pyrimidine Metabolism



CHAPTER 2. A STRATEGY FOR SELECTING MUTANTS DEFECTIVE IN WOUND INDUCTION IN *ARABIDOPSIS THALIANA*

A paper to be submitted to the *Plant Molecular Biology*

Lan Zhou[1] and Robert W. Thornburg[1,2]

Abstract

A negative selectable marker *codA* gene from *E. coli* was fused to potato wound-inducible proteinase inhibitor II (*pin*2) promoter and *Arabidopsis thaliana* plants were transformed. *Arabidopsis* nitrilase 1 (*nit*1) promoter fused to the *codA* gene was also transformed to *Arabidopsis* as a control. The *codA* gene encodes a cytosine deaminase that catalyzes the conversion of cytosine into uracil. The enzyme likewise converts non-toxic 5-fluorocytosine (5FC) to toxic 5-fluorouracil (5FU). Analysis of expression of the *pin*2-*codA* gene in transgenic leaves could be induced by several wound signals: sucrose, ABA, methyl jasmonate and mechanical wounding. Transgenic seedlings had dose-dependent 5FC sensitivity induced by methyl jasmonate. They could not survive at a level of higher than 500 μg/mL of 5FC. This level did not affect wild type seedlings. Therefore, *codA* can serve as a regulated selective marker in *Arabidopsis* for selecting mutants defective in wound signaling pathways. Homozygous transgenic seeds were mutagenized by EMS and selection on 5FC was performed in M2 and M3 seeds. Four M2 lines that had lost *codA* activity in M2 and M3 plants have been isolated. These potential mutants will be confirmed by genetic analysis.

Introduction

Plants, unlike animals, can not move when there are changes in the environment. Plants have evolved to adapt to these changes with a series of biochemical responses. Plants

---

*This work was sponsored by a grant (91-37301-6208) from the US Department of Agriculture.

[1]Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

[2]To whom correspondence should be addressed.

respond to wounding and pathogen attacks by activating a set of defense genes. Many of these defense genes are involved in wound-healing and prevention of subsequent pathogen invasion (Zhou and Thornburg, submitted). Some of these genes are activated in the damaged site (local) while others are induced to expressed in the distal undamaged parts (systemic) of plants (Green and Ryan 1972; Bowles 1990). Among these defensive proteins, two inhibitors of serine proteinase called Inhibitor I (Mr = 8100 KDa) (Ryan and Balls, 1962) and Inhibitor II (Mr = 12,300 KDa) (Bryant et al., 1976) were initially identified from tomato and potato. Proteinase inhibitors inhibit digestive proteinases in herbivorous insect guts to result in severe protein malnutrition, reduced growth and even death (Ryan, 1990). The potato proteinase inhibitor II (*pin*2) gene is well-characterized in systemic induction upon wounding (Bowles 1990; Ryan, 1990).

Several wound signals have been identified that induce proteinase inhibitor genes in plants. They are oligouronide fragments derived from plant cell walls (Bishop et al., 1984; Ryan, 1987), chitin and chitosan fragments from fungal cell walls (Walker-Simmons et al., 1983), an 18-amino-acid polypeptide from tomato leaves called systemin (Pearce et al., 1991), methyl jasmonate and jasmonic acid (Farmer and Ryan, 1990), abscisic acid (Peña-Cortés et al., 1989), auxin (Kernan and Thornburg, 1989), sucrose (Johnson and Ryan, 1990), ethylene (O'Donnell et al., 1996; Yen et al., 1995), electrical signals (Wildon et al., 1992) and hydraulic pulses (Malone et al., 1994).

It has been proposed that activation of defense genes is mediated by an octadecanoic acid-based signaling pathway in response to herbivore attack or other mechanical wounding (Farmer and Ryan, 1992a; Farmer and Ryan, 1992b). Several lines of evidence support this model. Linolenic acid, one of octadecanoid precursors, is released from intracellular lipid pools in plant suspension cultures treated with elicitor (Mueller et al., 1993) or mechanical wounding (Conconi et al., 1996). Application of jasmonic acid or its octadecanoid precursors activates proteinase inhibitor expression (Farmer and Ryan, 1992a). The synthesis of jasmonic

acid is induced by several wound signals including mechanical wounding (Creelman et al., 1992; Peña-Cortés et al., 1993; Blechert et al., 1995; Doares et al., 1995b), fungal elicitors (Gundlach et al., 1992), systemin (Doares et al., 1995b), oligosaccharides (Doares et al., 1995b), abscisic acid and octadecanoic acids (Peña-Cortés et al., 1995). Salicylic acid (Peña-Cortés et al., 1993; Doares et al., 1995a) and diethyldithiocarbamate (Farmer et al., 1994), two inhibitors of the octadecanoid pathway, block the activation of proteinase inhibitor genes. Transfer of an *Arabidopsis* lipoxygenase gene (encoding for the first enzyme in the conversion of linolenic acid to jasmonic acid) in transgenic plants results in inhibition of the accumulation of jasmonic acid and reduced levels of wound-inducible gene expression by cosuppression (Bell et al., 1995). Allene oxide synthase (CYP 74) from *Arabidopsis*, the first enzyme of the octadecanoid pathway to jasmonates has been cloned (Laudert et al., 1996). It has also been proposed that a second octadecanoid pathway exists in plants (Blechert et al., 1995; Laudert et al., 1996). In this pathway, dihydrojasmonate can be synthesized from linoleic acid via 15, 16-dihydro-12-oxo-phytodienoic acid.

A search for the receptor of systemin has led to the identification of a 50 KDa systemin-binding protein (SBP50) in tomato plasma membrane, which is not a receptor, but a Kex2p-like protease that can cleave systemin into smaller peptides (Schaller and Ryan, 1994). Residues in systemin for binding SBP50 seem to reside within the N-terminal and residues for proteinase inhibitor induction are located within the C-terminus. The residues for binding include a cleavage site for Kex2p-like protease family.

Genetic approaches to study wound-inducible signaling pathway have been also attempted and two mutants (JL1 and JL5) deficient in the activation of proteinase inhibitor I and II in response to wounding have been isolated from tomato (Lightner et al., 1993; Howe et al., 1996). It has been proposed that signaling step between the wound signal and the upstream production of JA is blocked in both mutants because they accumulated inhibitors when exposed to methyl jasmonate. Furthermore, JL5 mutant (*def*1) is defective in the signaling step after

lipoxygenase conversion of linolenic acid to hydroperoxylinolenic acid but before the conversion of PDA to JA (Howe et al., 1996). *Arabidopsis* mutants deficient in jasmonate perception have been isolated using coronatine, a chlorosis-inducing phytotoxin produced by several pathovars of *Pseudomonas syringae*. In tomato, coronatine induces the accumulation of proteinase inhibitors (Palmer and Bender, 1995). These mutants are resistant to coronatine and also insensitive to methyl jasmonate inhibition of root growth. Recently, a jasmonate binding protein that mediates the wound-inducible regulation of transcription of the potato proteinase inhibitor II gene has been identified (Gurevichet al., 1996). An inhibitor of some aminopeptidases in plants and animals, bestatin, has been identified as an inducer of proteinase inhibitor genes in tomato leaves (Schaller et al., 1995). Without causing an increase of intracellular jasmonic acid concentrations, its induction is not affected by inhibitors of the octadecanoid pathway. Furthermore, bestatin can induce defense genes in tomato JL5 mutant. Thus, bestatin appears to function close to the level of transcription of defense genes.

Knowledge about how proteinase inhibitor genes are induced has increased in recent years. However, the molecular basis of signal transduction pathways that regulate the expression of proteinase inhibitor genes still remains an unsolved problem. Traditional screening for mutants needs a simple and fast method to test a lot of mutagenized plants. In addition, mutation in an intermediate component of the signal transduction pathway may not cause a visible phenotype. For these reasons, we have developed an alternative and more directed genetic approach which allows us to directly select for mutants in wound-inducible pathway. The approach involves the fusion of the wound-inducible *pin*2 promoter to a negative selective marker gene *codA* and the stable transformation of *Arabidopsis* with the construct. The marker gene confers a phenotype that mutants deficient in wound response can survive the selection. This promoter fusion approach has been used in higher eukaryotes (Hofstetter et al., 1987; Pellegrini et al., 1989; Shirras and Bownes, 1989; Karlin-Neumann et al., 1991; Heimer et al., 1995), yeast (Struhl, 1983) and bacteria (Guarente et al., 1982).

In selecting negative marker genes for *Arabidopsis*, we have found a bacterial cytosine deaminase gene (*codA*) with a advantage over others. Because plants and mammals have no *codA* gene (Bendich et al., 1949; Ross, 1965; Stougaard, 1993). Thus, there is no background enzyme activity from the endogenous gene.

Cytosine deaminase catalyzes the conversion of cytosine to uracil. It also can catalyze non-toxic 5-fluorocytosine (5FC) to toxic 5-fluorouracil (5FU). Wild type plant cells have no *codA* gene, so they are insensitive to 5FC. By contrast, transgenic plant cells expressing cytosine deaminase are sensitive to 5FC. Therefore, *codA* gene is suitable for negative selection. *CodA* gene as a negative selective marker in plants has been successful in tobacco, *Lotus japonicus* (Stougaard, 1993) and *Arabidopsis* (Perera et al., 1993; Kobayashi, et al., 1995).

We previously have selected UMP synthase mutants from *Nicotiana plumbaginifolia* by 5-fluoroorotic acid (5FOA) negative selection (Santoso, 1995). These mutants are the knock-out mutants which have lost the expression of UMP synthase activity. Uracil permits rescue of these mutants by uracil phosphoribosyl transferase salvage pathway. We have made the construct *pin*2-UMPS^{Dd} (Shi and Thornburg, 1993), transformed the construct to *Nicotiana tabacum* and analyzed the expression of UMP synthase in transgenic plants at different levels (Zhou and Thornburg, unpublished). The data showed that *Dictyostelium discoideum* UMP synthase functions in plants. We attempted to transform the UMP synthase mutants with the construct *pin*2-UMPS^{Dd} and select for the second site mutations which block in wound induction by 5FOA negative selection. We had the problems with getting enough mutant materials for transformation. Unfortunately, we failed to regenerate these mutants to plants. They were in callus stage. The mutant calli grew very slowly and sometimes UMP synthase activity levels were not stable in the mutants. Using construct *pin*2-*codA* in our negative selection for mutants deficient in wound response has overcome the problems we had with UMP synthase mutants. Because plants do not have *codA* gene, we can transform the

construct directly to wild type plants and do the selection. It saves time and also makes the whole mutant selection process easier.

Arabidopsis nitrilase 1 and 2 promoters have been isolated and sequenced (Zhou et al. 1995; 1996). Nitrilase is the enzyme that converts IAN (indole acetonitrile) into auxin IAA (indole-3-acetic acid). It is a key enzyme in IAA biosynthesis. Four nitrilase genes have been isolated in *Arabidopsis* (Bartel and Fink, 1994). NIT1 is soluble and is expressed throughout development, while NIT2 is membrane-bound and is most strongly expressed during silique development (Bartling et al., 1994). The *nit*1 promoter is constitutive and *nit*2 promoter is inducible by infiltration of *Pseudomonas syringae* pv. *maculicola*, but it is not wound-inducible. We have made construct *nit*2-*codA* and transformed the construct to *Arabidopsis* (Zhou and Thornburg, unpublished). We are using the same 5FC negative selection strategy to isolate mutants blocked in activation of nitrilase 2 promoter. Construct *nit*1-*codA* serves as a non-regulated control, as it did in this report.

Here we report that the *codA* gene fused to the wound-inducible *pin*2 promoter can be regulated by wound signals and can behave as a conditional lethal marker for selection of mutants deficient in wound response in *Arabidopsis*.

Experimental Procedures

Plant materials, growth conditions and chemicals

Arabidopsis thaliana, ecotype *Columbia*, *Nicotiana tabacum* cv Xanthi and *Nicotiana tabacum* cv Xanthi transformed with a wound-inducible proteinase inhibitor II-CAT (*pin*2-CAT) construct Tr25 (Thornburg et al., 1987; Kernan and Thornburg, 1989) were used in these experiments. Seeds were surface sterilized in 70% ethanol for 2 min., 20% bleach/0.05% Tween 20 for 15 min and then rinsed with sterile water 5 times. The sterilized seeds were sown onto Murashige and Skoog medium (MS salts, 3% sucrose and 0.8% agar, pH 6.0) in either the of presence or absence of 50 μg/mL kanamycin for *Arabidopsis* or 200 μg/mL for *N. tabacum* , 5-fluorocytosine and 25 μM methyl jasmonate (Bedoukian Research,

Danbury, CT) as indicated. All plants were grown under standard greenhouse conditions with a long-day photoperiod (14 h light, 10 h dark). Restriction enzymes, T4 Ligase and Taq polymerase were from Promega (Madison, WI). All other enzymes and reagents were from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Oligonucleotides were synthesized at the Nucleic Acid Facility of Iowa State University. The bacterial strain XL1-Blue was used for all bacterial manipulations.

Construction of promoter fusions

To construct the *pin2-codA* gene fusion, the cytosine deaminase coding sequence and 5' untranslated tobacco mosaic virus Ω leader were amplified by PCR using the plasmid pNE3 (CaMV 35S-TMV Ω -*codA*, kindly provided by Dr. Jens Stougaard) as a template. A *Xba*I site and a *Nhe*I site were introduced by a N-terminal primer and a C-terminal primer, respectively. The 1.3 Kb PCR product was subsequently subcloned into pT7Blue(R) (Novagen) and eventually was linked with the *pin2* promoter and the *pin2* terminator to form the construct pRT348. The construct was then inserted into a plant transformation vector pGA482 (An, 1987) to form the construct pRT349 (Figure 1). The construct was confirmed by sequencing using a primer for *codA* and by Southern blot analysis using *codA* as a probe (Figure 1).

To construct the *nit1-codA* gene fusion, a 1.6 Kb nitrilase 1 promoter fragment was PCR amplified using nit36.3 (kindly provided by Dr. Bonnie Bartel) as a template and subcloned into pT7Blue(R) to form the construct pRT350. A *Sac*I site was introduced by a 3' end primer. The 1.6 Kb *Hin*dIII/*Sac*I fragment from pRT350 was excised and ligated with the 15.8 Kb *Hin*dIII/*Sac*I fragment from pRT349 to form the construct pRT354. The construct was confirmed by sequencing using a primer for *codA*.

Arabidopsis transformation

The construct pRT349 or pRT354 was transferred into *Agrobacterium tumefaciens* GV3101pM90 by the freeze-thaw method (Holsters et al., 1978). The *Agrobacterium* strain

was used to transform *Arabidopsis thaliana Columbia* by vacuum infiltration (Bechtold et al., 1993). Three to four weeks old and well developed plants in pots (5 or 6 plants per pot) were immersed in infiltration medium (1/2 X MS salts, 1 X Gamborg's B_5 vitamins, 5.0% sucrose, 0.044 μM benzylaminopurine) containing *Agrobacteria* under vacuum (< 100 Microns Hg) for 1 min. The plants were covered with saran wrap to maintain humidity for 2 days. After 4 weeks, seeds were collected, sterilized and plated out on selective medium (1/2 X MS salts, 0.8% agar, 1 X Gamborg's B_5 vitamins, 50 mg/L kanamycin). The seeds were vernalized for 2 nights in a cold room and moved to a growth chamber. The surviving plants then were transferred to soil and moved to a greenhouse.

Southern analysis

Genomic DNA was isolated from transgenic plants by the method of Dellaporta et al. (1983). The DNA was digested with *Hin*dIII/*Bgl*II which cut at each end of *pin2-codA -tpin2* or *nit1-codA-tpin2* within the construct and *Hin*dIII which cuts only once within the construct. The plasmid pRT349 or pRT354 digested with *Hin*dIII/*Bgl*II was used to mark the size. The DNA probe was the 1.3 Kb *Eco*RI/*Hin*dIII fragment of pRT336 labeled with α-[^{32}P]-dCTP by random primers. Southern blot analysis was carried out according to the method of Sambrook et al. (1989).

Cytosine deaminase assay

Cytosine deaminase assay was conducted according to the method of Stougaard (1993) as modified below. Cytosine deaminase assay is a crucial test in our experiments. In the protocol of Stougaard (1993), ten microliter of plant extract was used per assay. We found extract containing 100 μg proteins gave better results. But it is difficult to load so much extract on a TLC plate. Also it interfered TLC development. Thus, we modified the protocol by extracting reaction mix with 1-pentanol/ethyl acetate (1:1) after assay to get rid of other interfering materials from plant extract. This organic extraction recovered almost 100% of 2-^{14}C-cytosine and 2-^{14}C-uracil from the reaction (data not shown) and made loading and

development easier. Plant leaves were homogenized in extraction buffer (0.2 M Tris-HCl, pH 7.8, 1mM DTT, 1mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl and 2 mM PMSF). The homogenized juice was centrifuged at 10,000 g for 10 min and the supernatant was used for protein concentration determination by Bradford assay (Bradford, 1976). Ten microliters of assay buffer (0.2 M Tris-HCl pH 7.8, 1 mM DTT, 1 mM EDTA, 2 mM PMSF, 0.05 μCi 2-^{14}C- cytosine) was added to 100 μg protein of extracts. The assay was carried out at 37°C for 3 hours. The assay mix was made up to 100 μl with dH_2O and extracted with 1 ml of 1-pentanol/ethyl acetate (v/v, 1:1). The organic phase was dried. Twenty microliters of 1-pentanol/ethyl acetate (v/v 1:1) was added to each tube and spotted on a DC-Alufolien cellulose TLC plate (Alltech Associates, Inc., Deerfield, IL). The TLC plate was developed in 1-butanol/H_2O (v/v, 86:14) for 10 hours. The plate was dried, wrapped with saran wrap and autoradiographed. The position of uracil or cytosine on the TLC plate was determined by the location of unlabeled uracil or cytosine under short-wave ultraviolet light. For quatitation of cytosine deaminase activity, the spots containing 2-^{14}C- uracil and cytosine were counted in a scintillation counter. The enzyme activity was defined as the percent conversion of cytosine into uracil.

CAT assay

CAT assay was performed according to the method of Kernan and Thornburg (1989). Protein extracts were prepared by grinding the tissue in an equal volume of homogenization buffer (0.1 M Tris-HCl [pH 8.0], 0.5 M sucrose, 0.1% [w/v] ascorbic acid, 0.1% [w/v] cysteine HCl) in a Con-Torque homogenizer (Eberbach, Corp; Ann Arbor, MI). The homogenized juice was centrifuged for 10 min at 10,000 g and the supernatant was used for protein concentration determination by the method of Bradford (1976). The extract containing 100 μg was used for CAT assay using ^{14}C-chloramphenicol (100,000 cpm = 1.66 μmole) as substrate for 1 hr at 37°C. The reaction mix was extracted with 1 ml ethyl acetate. The organic layer was dried, taken up in 20 μl of ethyl acetate and spotted on a silica gel thin-layer plate

(Sigma). The TLC plate was run in chloroform/methanol (95:5). The CAT activity was visualized by exposure to a X-ray film. The TLC plates were overlayed onto the exposed film and the radioactive spots corresponding to labeled chloramphenicol acetate and unreacted chloramphenicol were cut out and counted in a scintillation counter. The enzyme activity was defined as the percent conversion of chloramphenicol into the acetylated forms of chloramphenicol.

Selection with 5-fluorocytosine and 5-fluorouracil

Approximately 500 to 1000 wild type or transgenic seeds of *Arabidopsis thaliana* or *Nicotiana tabacum* were surface sterilized and plated on MS media containing either 5-fluorocytosine or 5-fluorouracil at the indicated concentrations in either the presence or absence of 25 μM methyl jasmonate and 50 mg/L kanamycin. The plates were kept in a culture room (26°C, 14 h light, 10 h dark). After 12 days, the seedlings were scored for survival. The percent survival was defined as the number of surviving seedlings divided by the total number of seeds plated.

Induction of *pin*2 promoter

For the induction in seedlings, the seeds of Tr25 or Tr349 were surface sterilized and plated on MS media containing 3% sucrose, 100 mM abscisic acid, 1 mg/mL chitosan or 25 mM methyl jasmonate and 200 or 50 mg/L kanamycin. The seedlings were collected on the indicated days, stored at -700C and assayed together. For routine induction in leaves, a half leaf assay similar to the method of Kernan and Thornburg (1989) was used. One half of a leaf was incubated in MS liquid media containing 25 mM methyl jasmonate for Arabidopsis for 18-24 hr. The other half of leaf was stored at -700C as an uninduced sample. For the induction by mechanical wounding in leaves, leaves were wounded at several locations perpendicular to the main vein with a hemostat. After 18-24 hr, the wounded leaves were harvested.

Amplification of *Arabidopsis* seeds

For transgenic seeds, seeds were surface sterilized and plated on MS media containing 50 µg/mL kanamycin. After two weeks, the seedling were transferred to soil. For wild type seeds, seeds were planted on soil in a 10 cm pot covered with saran wrap. Saran wrap was removed once the seeds were germinated. Plantlets (about 1 cm tall) were separated and planted on soil. The plants were maintained in an ARASYSTEM growth and seed harvesting system (LEHLE SEEDS, Round Rock, TX) in a growth chamber (24°C, 14 h light, 10 h dark). The seeds were collected after 6 to 8 weeks when the plants were dry.

Mutagenesis

Seeds of *Arabidopsis* homozygous Tr349 lines were hydrated in distilled water overnight at 4°C and then resuspended in 0.1 M sodium phosphate (pH 7.0) containing 0.3% EMS (ethylmethane sulfonate) for 15 hr, followed by thorough washing with running water for 3 to 4 hr. The mutagen-treated seeds (M1) were suspended in 0.18% cold agar and 5 mL seed suspension containing 50 to 300 seeds was spread onto the surface of soil in 15 cm pots covered with saran wrap in greenhouse (Rédei, 1992). Saran wrap was removed when plantlets appeared. M2 seeds were harvested in bulk from M1 plants.

Screening

M2 seeds were surface sterilized as described above and plated on MS media containing 50 µg/mL kanamycin, 25 µM methyl jasmonate and 500 µg/mL 5-fluorocytosine. After about 2 weeks, the selected seedlings were transferred to soil. After 3 to 4 weeks, leaves were used for cytosine deaminase assay. M3 seeds were harvested from M2 plants. Rescreening was conducted in M3 seeds for 5-fluorocytosine resistance on the same medium described above. Seedlings were transferred to soil. After 3 to 4 weeks, leaves were used for cytosine deaminase assay. M4 seeds were harvested from M3 plants.

Results

Creation of homozygous *Arabidopsis* lines with *codA* constructs.

A negative selectable marker gene was constructed with either a construct containing a 0.9 Kb fragment from the potato *pin*2 promoter fused to the *codA* gene (*pin*2-*codA*, pRT349; corresponding transgenic plants are termed Tr349) or a construct containing a 1.6 Kb *Arabidopsis* nitrilase 1 promoter fragment fused to the *codA* gene (*nit*1-*codA*, pRT354; corresponding transgenic plants are termed Tr354). The *nit*1-*codA* construct served as a non-wound-inducible control. Both constructs were confirmed by sequencing. Southern blot using *codA* gene as a probe also showed the correctness of construct pRT349 (Figure 1).

Both of the construct pRT349 and pRT354 were individually transferred to *Argobacterium tumefaciens* GV3101pM90 and then transformed into *Arabidopsis*. Genomic DNA gel blot analysis with the *codA* gene as probes indicated that the *codA* fusion constructs were intact in the transgenic lines (Figure 2). The *Hin*dIII/*Bgl*II digestion showed the presence of *codA* gene and the *Hin*dIII digestion showed the copy number of *codA* in the genome of that plant. DNA from untransformed plants did not hybridize to the *codA* gene, as evidenced by the lack of bands in both digestions. All Tr349 or Tr354 transgenic plants showed a single band corresponding to the size of *pin*2-*codA*-t*pin*2 (3.2 Kb) or *nit*1-*codA*-t*pin*2 (3.9 Kb) in *Hin*dIII/*Bgl*II digestions. Tr349#1, #3, #4 and #6 plant contained 4, 3, 6 and 2 copies of construct, respectively. While Tr354#1, #2 and #19 plant contained 4, 2 and 2 copies of construct, respectively.

Homozygous plants were obtained from representative lines transformed with either construct based on kanamycin resistance in their progeny. These transgenic lines were used for further analysis.

Wound-inducible or constitutive cytosine deaminase activity is detected in transgenic plants.

Leaves from three to four week old plants were tested for cytosine deaminase activity in transgenic Tr349 and Tr354 plants. The Tr349 leaves were treated with sucrose, methyl jasmonate, ABA or mechanical wounding for the *pin*2 induction. Figure 3 showed that

cytosine deaminase activity in Tr349#1 plant was induced 10 to 20 fold by one treatment compared to the activity level in untreated leaves. Similar results were also observed in other Tr349 plants. By contrast, in the Tr354 plants, the enzyme activity was expressed constitutively at high levels (Figure 4). Wild type leaves were never found to have the detectable enzyme activity under any treatments tested. Thus, cytosine deaminase activity levels are positively regulated by *pin*2 induction in Tr349 plants.

Methyl jasmonate induces *pin*2 promoter to function in *Arabidopsis* Tr349 seedlings.

In order to select mutants at the seedling stage, *pin*2 induction conditions had to be defined before selection. *Nicotiana tabacum* Tr25 seedlings (previously well-studied for *pin*2 induction in leaves. Thornburg et al., 1987; Kernan and Thornburg, 1989) were first tested for the induction by several chemicals known to induce the *pin*2 promoter (Figure 5). After plating on inducing media, some of the seedlings were collected each day for 10 days and assayed for CAT activity. The results showed that neither sucrose nor ABA induced *pin*2 in seedlings. Chitosan gave a weak induction (5% activity) on the 9th day. But methyl jasmonate-treated seedlings were induced on the 7th day (10% activity) and CAT activity increased afterwards. Therefore, methyl jasmonate was the best inducer for *pin*2 induction in tobacco seedlings among the chemical inducers tested here. Later methyl jasmonate was tested for *pin*2 induction in *Arabidopsis* Tr349 seedlings. After plating on inducing media, some of the seedlings were collected each day for 20 days and assayed for cytosine deaminase activity (Figure 6). The results showed that methyl jasmonate started induction on the 3rd day (36% activity). This is earlier than that in the tobacco transformants. Furthermore, the induction lasted till the 20th day tested. Also, the induction was strong (about 90% activity). After the 20th day, the induction declined (data not shown). Similar results were also found with Tr349#3 seedlings. The results indicate that *pin*2 promoter can function in *Arabidopsis*, as it does in solanaceous plants. Cytosine deaminase was almost fully active for 17 days after the induction. This

timeframe provides sufficient time for selection before the enzyme activity declines. Thus, the selection of mutants should be done before the 21st day of induction.

Expression of the *codA* gene confers 5-fluorocytosine sensitivity on Tr349 transgenic seedlings.

The other conditions for the selection of mutants were also tested, namely the effect of 5FC or 5FU at various concentrations on survival of wild type *Arabidopsis thaliana* and *Nicotiana tabacum* seedlings (Figure 7). Figure 7A showed that 5FU had a dose-dependent effect on wild type seedlings of both species. The seedlings could not survive at a level of 5FU higher than 100 and 130 μg/mL, respectively. However, they could survive at 5FC levels as high as 1 mg/mL (Figure 7B). Therefore, expression of *codA* gene was expected to result in toxicity of 5FC.

Effect of 5FC on survival of seedlings was also tested in *Arabidopsis* transgenic Tr349 and Tr354 seedlings (Figure 7B). Figure 7B clearly showed that Tr349 seedlings on 5FC without *pin*2 induction by methyl jasmonate behaved like wild type seedlings. There was no effect of 5FC at a level as high as 1 mg/mL. However, when Tr349 seedlings grew under *pin*2 induction conditions, their survival was greatly dependent on 5FC concentrations. The seedlings died at 500 μg/mL of 5FC. Similarly, Tr354 seedlings harboring constitutively expressed cytosine deaminase activity were nearly completely killed by 5FC at a level of 500 μg/mL. This result indicated that *codA* gene expression is regulated by *pin*2 induction. Methyl jasmonate can induce *pin*2 promoter in Tr349 to drive *codA* which results in sensitivity to 5FC. The concentration of 5FC, 500 μg/mL, killed all the Tr349 seedlings with *pin*2 induction. This concentration was used for further mutant selections. Because these plants were expressing the *codA* gene in an appropriate manner, Tr349#1 and #3 plants were chosen for selection.

Mutagenesis and screening

Figure 8 outlines the scheme used for mutant selection. About 6.8 X 10^5 Tr349#1 and 2.0 X10^5 Tr349#3 were mutagenized by EMS and planted in soil to generate M1 plants. About

2.1 X 10^5 M2 seeds from Tr349#1 M1 plants and 4.5 X 10^4 M2 seeds from Tr349#3 M1 plants were screened on selective media as described in the "Experimental Procedures". Thirty seedlings from Tr349#1 M2 seeds and 80 seedlings from Tr349#3 M2 seeds survived on the selective media. However, most of these surviving plants were killed in a power failure accident before their M3 seeds could be collected. Finally, two M2 plants of Tr349#1 and 18 M2 plants of Tr349#3 produced M3 seeds. Approximately 1,000 M3 seeds (100 from Tr349#1 and 900 from Tr349#3) were rescreened on selective media to eliminate false positives. Ninety three seedlings were grown from 900 M3 seeds of Tr349#3, but there were no M3 plants selected from Tr349#1 M3 seeds. These 93 M3 plants belonged to 4 different mutant lines of original 18 M2 plants of Tr349#3. Out of 93 plants in soil, leaves from 24 plants of these 4 mutant lines were induced by methyl jasmonate and assayed for cytosine deaminase activity. No activity (< 0.5%) was found in these 24 plants (Figure 9). As a control, a parental plant of Tr349#3 T5 without mutagenesis was assayed at the same time and the enzyme activity (30%) was detected. Similar results were obtained when the enzyme activity was tested in their M2 plants (data not shown). The enzyme assay result indicated that the *codA* gene was not expressed to produce a functional enzyme in these mutant plants. The mutants may be unable to turn on *pin*2 promoter as expected. However, they may have mutations in *pin*2 or *codA* gene itself. Thus, further genetic analysis need to be performed in these mutants.

Discussion

The experiments presented here demonstrate the feasibility of the bacterial *codA* gene as a selectable marker gene for negative selection in *Arabidopsis*. The *codA* gene encodes cytosine deaminase activity which is not present in wild type plants. Transgenic seedlings containing the construct *nit*1-*codA*, constitutively expressing *codA* gene, were affected by concentrations of 5FC as low as 100 µg/mL. 5FC even at 1 mg/mL had no effect on seedlings of both wild type and Tr349 without *pin*2 induction by methyl jasmonate. However, no

seedlings of either Tr349 with *pin*2 induction or Tr354 survived at 500 μg/mL of 5FC. Therefore, the concentration of 5FC, 500 μg/mL, was used for all the selections. The data also indicated that construct *nit*1-*codA* is a good non-regulated control.

The potato proteinase inhibitor II promoter *pin*2 was used to drive the *codA* expression in *Arabidopsis* transgenic plants. Prior to these studies, it was believed that the wound-inducible potato *pin*2 promoter does not function in *Arabidopsis*. This report is the first demonstration that potato *pin*2 promoter is functional in wound-inducible manner in *Arabidopsis*.

Because methyl jasmonate was able to induce the *pin*2 promoter in seedlings, mutant selection in seedlings is possible. To conduct such selections, seedlings needed to be kept on selective media for 2 to 3 weeks. However, methyl jasmonate is volatile. Our previous studies with the tobacco transgenic seedlings containing the construct *pin*2-*codA* showed that *codA* activity induced by methyl jasmonate began to decline after the 10th day of induction (data not shown). Therefore, additional methyl jasmonate was added to each plate on the 10th day of induction. This additional application of methyl jasmonate proved effective in maintaining a sufficient methyl jasmonate concentration to drive the *pin*2-*codA* gene in *Arabidopsis* seedlings (Figure 6). Our data also indicated that methyl jasmonate on selective media inhibited seed germination as reported previously (Staswick, 1992). It was observed that the seeds on selective media began to germinate 3 to 4 days later than those seeds on the media without methyl jasmonate. The seedlings grew normally although slowly once the seeds germinated. However, the surviving seedlings on the media were still distinguishable during the timeframe of selection.

We have used the 5FC negative selection strategy and identified 4 mutant lines which do not have inducible *codA* activity. In order to determine the genetic basis for the defects in wound signaling in these lines, crosses will be conducted between each mutant and the parental transgenic plant and between two mutants. Other kinds of mutations that might be selected in

this screen include methyl jasmonate resistance mutants, *cis*-mutants (mutation on *pin2-codA*) and metabolic mutants (affecting general pyrimidine metabolism). But these should be easily distinguishable. In our experiments, we used methyl jasmonate to induce the *pin2* promoter. The mutant blocked in the *pin2* induction by methyl jasmonate does not necessarily have a defect in the induction by wounding because it is possible that several alternative signaling pathways might exist.

Acknowledgements

We gratefully thank Dr. Jens Stougaard from Laboratory of Gene expression, Department of Molecular Biology, University of Aarhus, Denmark for providing us with the pNE3, Dr. Dan Voytas from Iowa State University for the *Agrobacterium* host strain GV3101pM90 and Dr. Bonnie Bartel from Rice university for Nitrilase 1 and 2 genes.

References

An, G. (1987) Binary vectors. Methods Enzymol. 153, 292-305.

Bartel, B., and Fink, G.R. (1994) Differential regulation of an auxin-producing nitrilase gene family in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 91, 6649-6653.

Bartling, D., Seedorf, M., Schmidt, R.C. and Weiler, E.W. (1994) Molecular characterization of two cloned nitrilases from *Arabidopsis thaliana*: Key enzymes in biosynthesis of the plant hormone indole-3-acetic acid. Proc. Natl. Acad. Sci. USA 91, 6021-6025.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) *In planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. 316, 1194-1199.

Bell, E., Creelman, R.A. and Mullet, J.E. (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 92, 8675-8679.

Bendich, A., Getler, H. and Brown, G. (1949) A synthesis of isotopic cytosine and a study of its metabolism in rats. J. Biol. Chem. 177, 565-570.

Bishop, P.D., Pearce, G., Bryant, J.E. and Ryan, C.A. (1984) Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves. J. Biol. Chem. 259, 13172-13177.

Blechert, S., Brodschelm, W., Holder, S., Kammerer, L., Kutchan, T.M., Mueller, M.J., Xia, Z.Q. and Zenk, M.H. (1995) The octadecanoid pathway: Signal molecules for the regulation of secondary pathways. Proc. Natl. Acad. Sci. USA 92, 4099-4105.

Bowles, D. (1990) Defense-related proteins in higher plants. Annu. Rev. Biochem. 59, 873-907.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72, 248-254.

Bryant, J., Green, T., Gurusaddaiah, T. and Ryan, C.A. (1976) Proteinase inhibitor II from potatoes: Isolation and characterization of the isoinhibitor subunits. Biochemistry 15, 3418-3423.

Conconi, A., Miquel, M., Browse, J.A. and Ryan, C.A. (1996) Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. Plant Physiol. 111, 797-803.

Creelman, R.A., Tierney, M.L. and Mullet, J.E. (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. USA 89, 4938-4941.

Dellaporta, S., Wood, J. and Hicks, J.B. (1983) A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1, 19-21.

Doares, S.H., Narvaez-Vasquez, J., Conconi, A .and Ryan, C.A. (1995a) Salicylic acid inhibits synthesis of poteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol. 108, 1741-1746.

Doares, S.H., Syrovets, T., Weiler, E.W. and Ryan, C.A. (1995b) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc. Natl. Acad. Sci. USA 92, 4095-4098.

Farmer, E.E. and Ryan, C.A. (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc. Natl. Acad. Sci. USA 87, 7713-7716.

Farmer, E.E. and Ryan, C.A. (1992a) Octadecanoid precursors of jasmonic acid activate synthesis of wound-inducible proteinase inhibitors. Plant Cell 4, 129-134.

Farmer, E.E. and Ryan, C.A. (1992b) Octadecanoid-derived signals in plants. Trends Cell Biol. 2, 236-241.

Farmer, E.E., Caldelari, D., Pearce, G., Walker-Simmons, M.K. and Ryan, C.A. (1994) Diethyldithiocarbamic acid inhibits the octadecanoid signaling pathway for the wound induction of proteinase inhibitors in tomato leaves. Plant Physiol. 106, 337-342.

Green, T.R. and Ryan, C.A. (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175, 776-777.

Guarente, L., Nye, J.S., Hochschild, A. and Ptashne, M. (1982) Mutant λ phage repressor with a specific defect in its positive control function. Proc. Natl. Acad. Sci. USA 79, 2236-2239.

Gundlach, H., Maller, M.J., Kutchan, T.M. and Zenk, M.H. (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA 89, 2389-2393.

Gurevich, A.I., Tuzova, T.P., Shpak, E.D., Starkova, N.N., Esipov, R.S. and Miroshnikov, A.I. (1996) Mechanism of action of the plant hormone jasmonate. 1. Jasmonate-interacting proteins that regulate transcription of the p. *pin*II potato gene. Bioorg. Khim. 22, 101-107.

Heimer, Y.M., Brusslan, J.A., Kenigsbuch, D. and Tobin, E.M. (1995) A chimeric *Lhcb::Nia* gene: an inducible counter selection system for mutants in the phytochrome signal transduction pathway. Plant Mol. Biol. 27, 129-136.

Hofstetter, P., Kikinis, Z., Altus, M.S., Pearson, D. and Nagamine, Y. (1987) A new genetic approach for studying hormonal regulation of urokinase-type plasminogen activator gene expression in LLC-PK1 cells. Mol. Cell. Biol. 7, 4535-4541.

Holsters, M., de Waele, D., Depicker, A., Messens, E., Van Montagu, M. and Schell, J. (1978) Transfection and transformation of *A. tumefaciens*. Mol. Gen. Genet. 163, 181-187.

Howe, G.A., Lightner, J., Browse, J. and Ryan, C.A. (1996) An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. Plant Cell 8, 2067-2077.

Johnson, R and Ryan, C.A. (1990) Wound-inducible potato inhibitor II genes: Enhancement of expression by sucrose. Plant Mol. Biol. 14, 527-536.

Karlin-Neumann, G., Brusslan, J.A. and Tobin, E.M. (1991) Phytochrome control of the *tms*2 gene in transgenic *Arabidopsis*: A strategy for selecting mutants in the signal transduction pathway. Plant Cell 3, 573-582.

Kernan, A. and Thornburg, R.W. (1989) Auxin levels regulate the expression of a wound-inducible proteinase inhibitor II-chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. Plant Physiol. 91, 73-78.

Kobayashi, T., Hisajima, S., Stougaard, J. and Ichikawa, H. (1995) A conditional negative selection for *Arabidopsis* expressing a bacterial cytosine deaminase gene. Jpn. J. Genet. 70, 409-422.

Laudert, D., Pfannschmidt, U., Lottspeich, F., Hollander-Czytko, H. and Weiler, E.W. (1996) Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol. Biol. 31, 323-335.

Lightner, J. Pearce, G., Ryan, C.A. and Browse, J. (1993) Isolation of signaling mutants of tomato (*Lycopersicon esculentum*). Mol. Gen. Genet. 241, 595-601.

Malone, M., Alarcon, J.J. and Palumbo, L. (1994) An hydraulic interpretation of rapid, long-distance wound-signaling in the tomato. Planta 193, 181-185.

Mueller, M.J., Brodschelm, W., Spannagl, E. and Zenk, M.H. (1993) Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc. Natl. Acad. Sci. USA 90, 7490-7494.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, D.J. (1996) Ethylene as a signal mediating the wound response of tomato plants. Science 274, 1914-1917.

Palmer, D.A. and Bender, C.L. (1995) Ultrastructure of tomato leaf tissue treated with the pseudomonad phytotoxin coronatine and comparison with methyl jasmonate. Mol. Plant Microb. interact. 8, 683-692.

Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 253, 895-898.

Pellegrini, S., John, J., Shearer, M., Kerr, I.M. and Stark, G.R. (1989) Use of a selectable marker regulated by alpha-interferon to obtain mutations in the signaling pathway. Mol. Cell Biol. 9, 4605-4612.

Peña-Cortés, H., Albrecht, T., Prat, S., Weiler, E.W. and Willmitzer, L. (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta 191, 123-128.

Peña-Cortés, H., Fisahn, J. and Willmitzer, L. (1995) Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. Proc. Natl. Acad. Sci. USA 92, 4106-4113.

Peña-Cortés, H., Sanchez-Serrano, J., Rocha-Sosa, M. and Willmitzer, L. (1989) Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc. Natl. Acad. Sci. USA 86, 9851-9855.

Perera, R.J., Linard, C.G. and Singer, E.R. (1993) Cytosine deaminase as a negative selective marker for *Arabidopsis*. Plant Mol. Biol. 23, 793-799.

Rédei, G.P. (1992) Classical mutagenesis in "Methods in *Arabidopsis* Research", Chapter 2, 16-73, edited by Koncz, C., Chua, N.-H., and Schell, J., World Scientific, River Edge, Singapore.

Ross, C. (1965) Comparison of incorporation and metabolism of RNA pyrimidine nucleotide precursors in leaf tissues. Plant Physiol. 40, 65-73.

Ryan, C.A. (1987) Oligosaccharide signaling in plants. Annu. Rev. Cell Biol. 3, 295-317.

Ryan, C.A. (1990) Proteinase inhibitor genes in plants: Genes for improving defenses against insects and pathogens. Annu. Rev. Phytopath. 28, 425-449.

Ryan, C.A. and Balls, A.K. (1962) An inhibitor of chymotrypsin from *Solanum tuberosum* and its behavior toward trypsin. Proc. Natl. Acad. Sci. USA 48, 1839-1844.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Santoso, D. (1995) 5-fluoroorotic acid-selected cell lines and regulation of UMP synthase gene expression in tobacco cells. Ph.D. thesis. Iowa State University, Ames.

Schaller, A. and Ryan, C.A. (1994) Identification of a 50 KDa systemin-binding protein in tomato plasma membrane having Kex2p-like properties. Proc. Natl. Acad. Sci. USA 91, 11802-11806.

Schaller, A., Bergey, D.R. and Ryan, C.A. (1995) Induction of wound response genes in tomato leaves by bestatin, an inhibitor of aminopeptidases. Plant cell 7, 1893-1898.

Shi, N.Q. and Thornburg, R.W. (1993) Construction of a UMP synthase expression cassette from *Dictyostelium discoideum*. Gene. 127, 199-202.

Shirras, A.D. and Bownes, M. (1989) Cricklet: A locus regulating a number of adult functions of *Drosophila melangaster*. Proc. Natl. Acad. Sci. USA 86, 4559-4563.

Staswick, P.E. (1992) Jasmonate, genes, and fragrant signals. Plant physiol. 99, 804-807.

Stougaard, J. (1993) Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene. Plant J. 3, 755-761.

Struhl, K. (1983) The new yeast genetics. Nature 305, 391-397.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R. and Ryan, C.A. (1987) Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyl transferase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA 84, 744-748.

Walker-Simmons, M., Hadwiger, L. and Ryan, C.A. (1983) Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves. Biochem. Biophys. Res. Comm. 110, 194-199.

Wildon, D.C., Thain, J.F., Minchin, P.E.H., Gubb, I.R., Reilly, A.J., Skipper, Y.D., Doherty, H.M., O'Donnell, P.J. and Bowles, D.J. (1992) Electrical signaling and systemic proteinase inhibitor induction in the wounded plant. Nature 360, 62-65.

Yen, H.C., Lee, S., Tanksley, S.D., Lanahan, M.B., Klee, H.J. and Giovannoni, J.J. (1995) The tomato *Never-ripe* locus regulates ethylene-inducible gene expression and is linked to a homolog of the *Arabidopsis ETR*1 gene. Plant Physiol. 107, 1343-1353.

Zhou, L. and Thornburg, R.W. Wound-inducible genes in plants (in press).

Zhou, L., Bartel, B. and Thornburg, R.W. (1995) Nucleotide sequence of the *Arabidopsis thaliana* nitrilase 1 gene (Accession No. U38845) (PGR95-130). Plant Physiol. 110, 337.

Zhou, L., Bartel, B. and Thornburg, R.W. (1996) Nucleotide sequence of a pathogen induced nitrilase gene from *Arabidopsis thaliana*: Nit2 (Accession No. U47114) (PGR96-006). Plant Physiol 110, 1048.

Figure Legends

Figure 1. Southern blot analysis of the construct pRT349.

Panel A, agarose gel. **Panel B**, Southern blot. Lane1, *Hin*dIII/*Eco*RI digestion; Lane 2, *Hin*dIII/*Bgl*II digestion; Lane 3, *Sac*I/*Bgl*II digestion; Lane 4, λ DNA digested with *Hin*dIII. The DNA probe was synthesized from *codA* coding region by random primers. 5.9 Kb, 3.2 Kb and 2.3 Kb bands were detected in Lane 1, 2 and 3 in **Panel B**, respectively.

Figure 2. Southern blot analysis of *Arabidopsis* transgenic Tr349 and Tr354 plants.

Genomic DNA was isolated from the *Arabidopsis* leaves of wild type, Tr349 and Tr354 plants, then digested with *Hin*dIII/*Bgl*II and *Hin*dIII, respectively. The DNA probe was synthesized from *codA* coding sequence (1.3 Kb) by random primers. Ten micrograms of leaf DNA was loaded per lane. **Panel A**, Tr349 plants. Lane 1 and 2, wild type; Lane 3 and 4, Tr349#1; Lane 5 and 6, Tr349#3; Lane 7 and 8, Tr349#4; Lane 9 and 10, Tr349#6 and Lane 11, plasmid pRT349. Lane 1, 3, 5, 7 and 9, *Hin*dIII digestion. Lane 2, 4, 6, 8, 10 and 11, *Hin*dIII/*Bgl*II digestion. **Panel B**, Tr354 plants. Lane 1 and 2, wild type; Lane 3 and 4, Tr354#1; Lane 5 and 6, Tr354#2; Lane 7 and 8, Tr354#19 and Lane 9, plasmid pRT354. Lane 1, 3, 5 and 7, *Hin*dIII digestion; Lane 2, 4, 6, 8 and 9, *Hin*dIII/*Bgl*II digestion.

Figure 3. Cytosine deaminase activity in the uninduced and induced *Arabidopsis* wild type and Tr349 plants.

Total protein was extracted from the *Arabidopsis* leaves of wild type and Tr349#1 plants. One hundred micrograms of total leaf protein was assayed. In each reaction 0.05 μCi 2-^{14}C-cytosine was used. After 3 h incubation at 37°C, the reaction mix was extracted with 1-pentanol/ethyl acetate (1:1). The organic phase was dried and loaded on a DC-Alufolien cellulose TLC plate. **Panel A**, induced by 3% sucrose; **Panel B**, induced by 25 μM methyl jasmonate; **Panel C**, induced by mechanical wounding and **Panel D**, 100 μM ABA. Lane 1 and 2: wild type; Lane 3 and 4: Tr349#1; Lane1 and 3: uninduced; Lane 2 and 4: induced.

Figure 4. Cytosine deaminase activity in *Arabidopsis* wild type and Tr354 plants.

Total protein was extracted from the *Arabidopsis* leaves of wild type, Tr354#1, Tr354#2 and Tr354#19 plants. One hundred micrograms of total leaf protein was assayed. In each reaction 0.05 μCi 2-^{14}C-cytosine was used. After 3 h incubation at 37°C, the reaction mix was extracted with 1-pentanol/ethyl acetate (1:1). The organic phase was dried and loaded on a DC-Alufolien cellulose TLC plate. Lane 1, wild type; Lane 2, Tr354#1; Lane 3, Tr354#2 and Lane 4, Tr354#19.

Figure 5. Time course of induction of CAT activity in *N. tabacum* Tr25 seedlings.

Tr25 seeds were surface-sterilized and plated on MS media containing 200 mg/L kanamycin and 3% sucrose, 100 μM ABA, 1 mg/mL chitosan or 25 μM methyl jasmonate. The samples were collected on the indicated days and frozen at -70°C. All the samples were assayed together. One hundred micrograms of total protein was assayed for CAT activity.

Figure 6. Time course of induction of cytosine deaminase activity in *Arabidopsis* Tr349 seedlings by methyl jasmonate.

Tr349#1 seeds were surface-sterilized and plated on MS media containing 50 mg/L kanamycin and 25 μM methyl jasmonate. The samples were collected on the indicated days and frozen at -70°C. All the samples were assayed together. One hundred micrograms of total protein was assayed for cytosine deaminase activity. **Panel A,** an autoradiogram showing expression of cytosine deaminase activity at the indicated days. **Panel B,** Quantitation of cytosine deaminase activity at the indicated days.

Figure 7. Survival of wild type seedlings of *Arabidopsis* and *N. tabacum* and Transgenic Tr349 and Tr354 seedlings of *Arabidopsis* on fluoropyrimidines.

About 500-1000 seeds were surface-sterilized and plated on MS media containing either 5-fluorocytosine or 5-fluorouracil at the indicated concentrations. After 12 days, the seedlings

were scored for survival. **Panel A**, wild type seedlings on 5-fluorouracil. ■ and ▲ represent *N. tabacum and Arabidopsis* , respectively. **Panel B**, wild type and transgenic seedlings on 5-fluorocytosine. × , wild type of *N. tabacum*; Δ, wild type of *Arabidopsis*; ○, *Arabidopsis* Tr349 on MS (50 mg/L Kanamycin + 5-fluorocytosine); ●, *Arabidopsis* Tr349 on MS (50 mg/L kanamycin + 25 μM methyl jasmonate + 5-fluorocytosine) and ■, *Arabidopsis* Tr354 on MS (50 mg/L Kanamycin + 5-fluorocytosine).

Figure 8. Schematic diagram of mutant selection.

Homozygous Tr349#1 or Tr349#3 seeds were used for mutagenesis. Selection was conducted on MS media containing 50 μg/mL kanamycin + 25 μM methyl jasmonate + 500 μg/mL 5FC. Leaves from 3 to 4 week old plants were used for cytosine deaminase assay.

Figure 9. Cytosine deaminase activity in four mutant lines of M3 plants of *Arabidopsis* Tr349#3.

Total protein was extracted from the *Arabidopsis* leaves of Tr349#3 T5 plants and four mutant lines of Tr349#3 M3 plants. One hundred micrograms of total leaf protein was assayed. In each reaction 0.05 μCi 2-^{14}C-cytosine was used. After 3 h incubation at 37°C, the reaction mix was extracted with 1-pentanol/ethyl acetate (1:1). The organic phase was dried and loaded on a DC-Alufolien cellulose TLC plate. Lane 1 and 2, Tr349#3 T5; Lane 3 and 4, 5 and 6, 7 and 8, 9 and 10 represent mutant line 1, 4, 6, and 7, respectively. Lane 1, 3, 5, 7 and 9, uninduced; Lane 2, 4, 6, 8 and 10, induced.

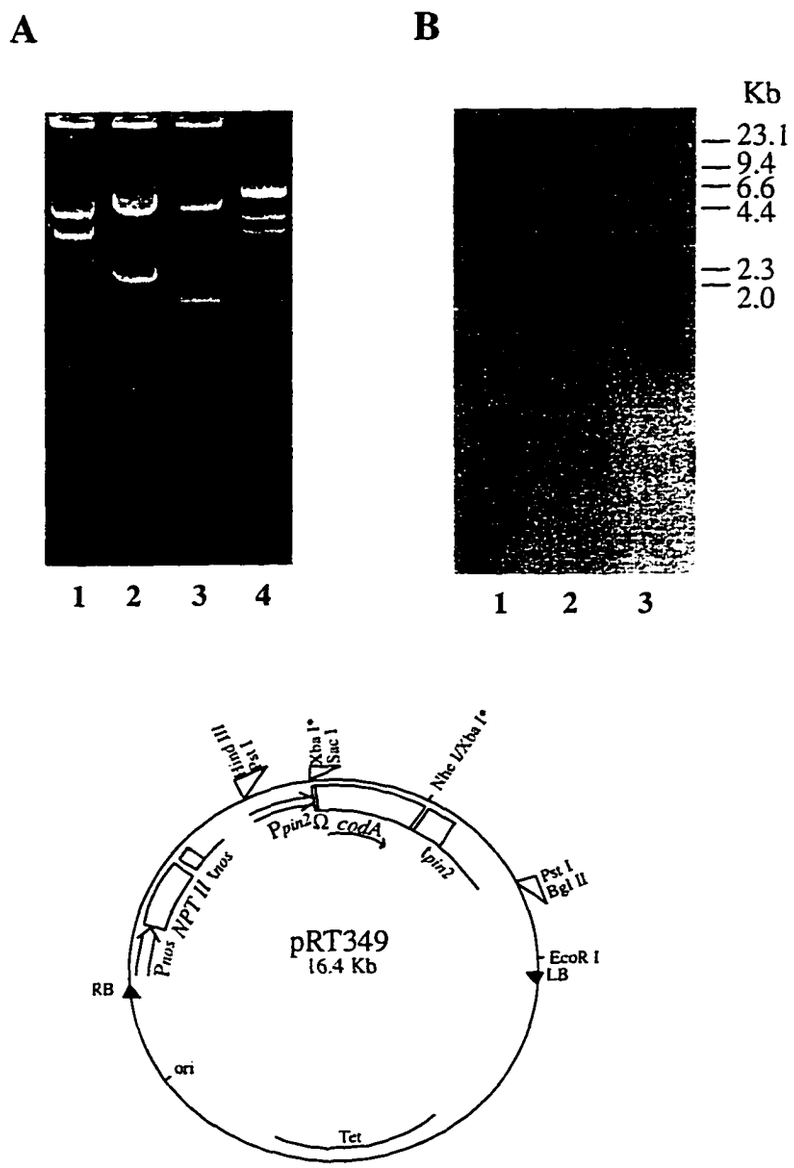
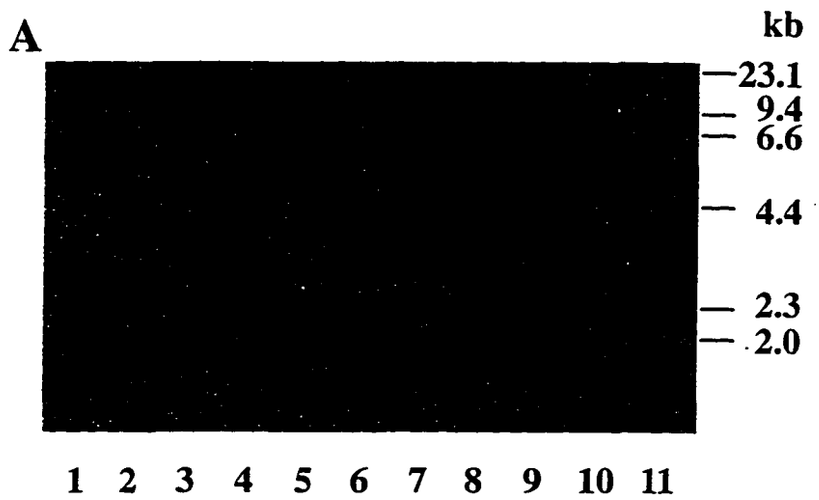

A
B
Kb
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3 4
1 2 3
pRT349
16.4 Kb
RB
LB
ori
Tet
EcoR I
Pst I
Bgl II
Nhe I/Xba I
Xba I
Sac I
Sal I
Hind III
Pnos NPT II tnos
Ppin2 Ω codA
tpin2

Figure 1


A
kb
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3 4 5 6 7 8 9 10 11

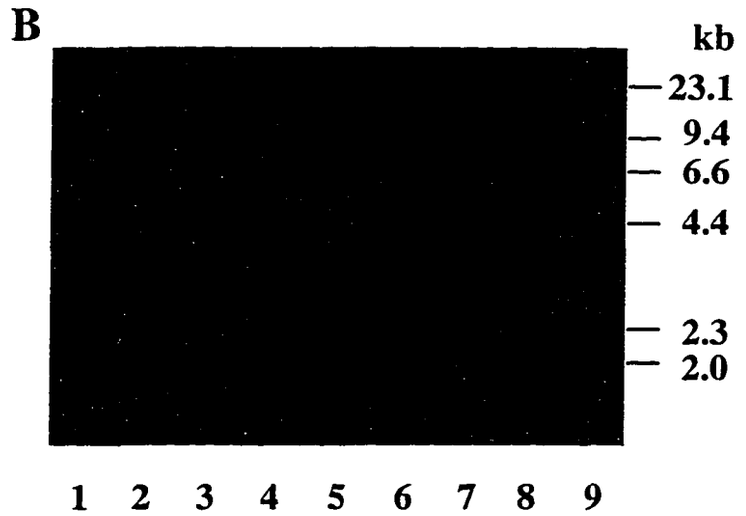

B
kb
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3 4 5 6 7 8 9

Figure 2

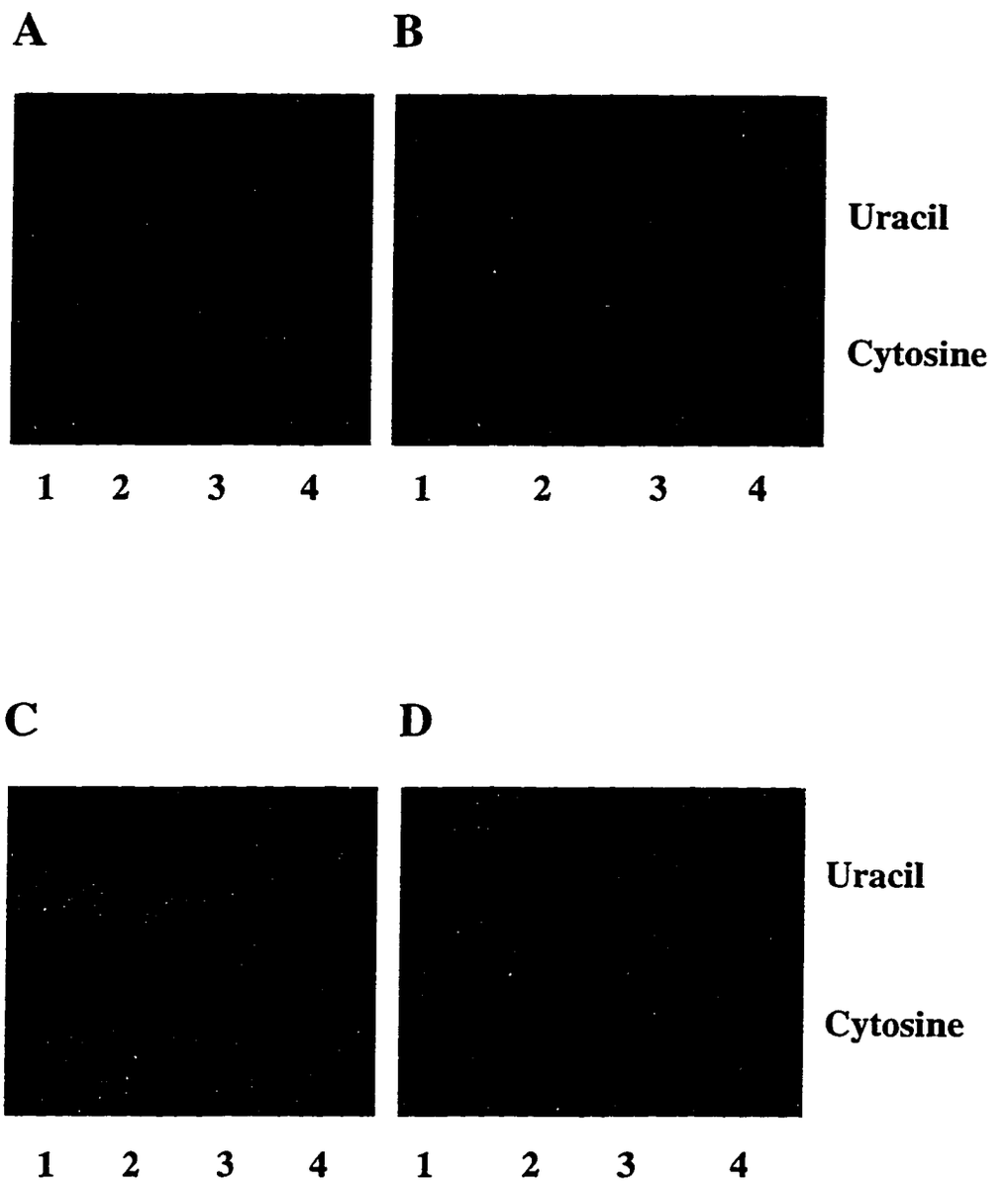


Figure 3

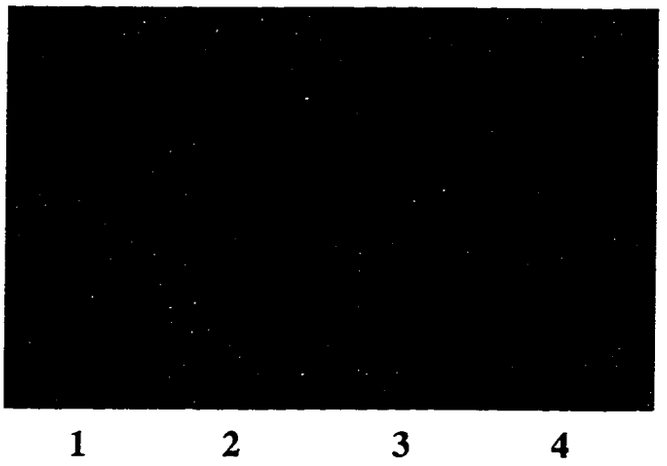


Figure 4

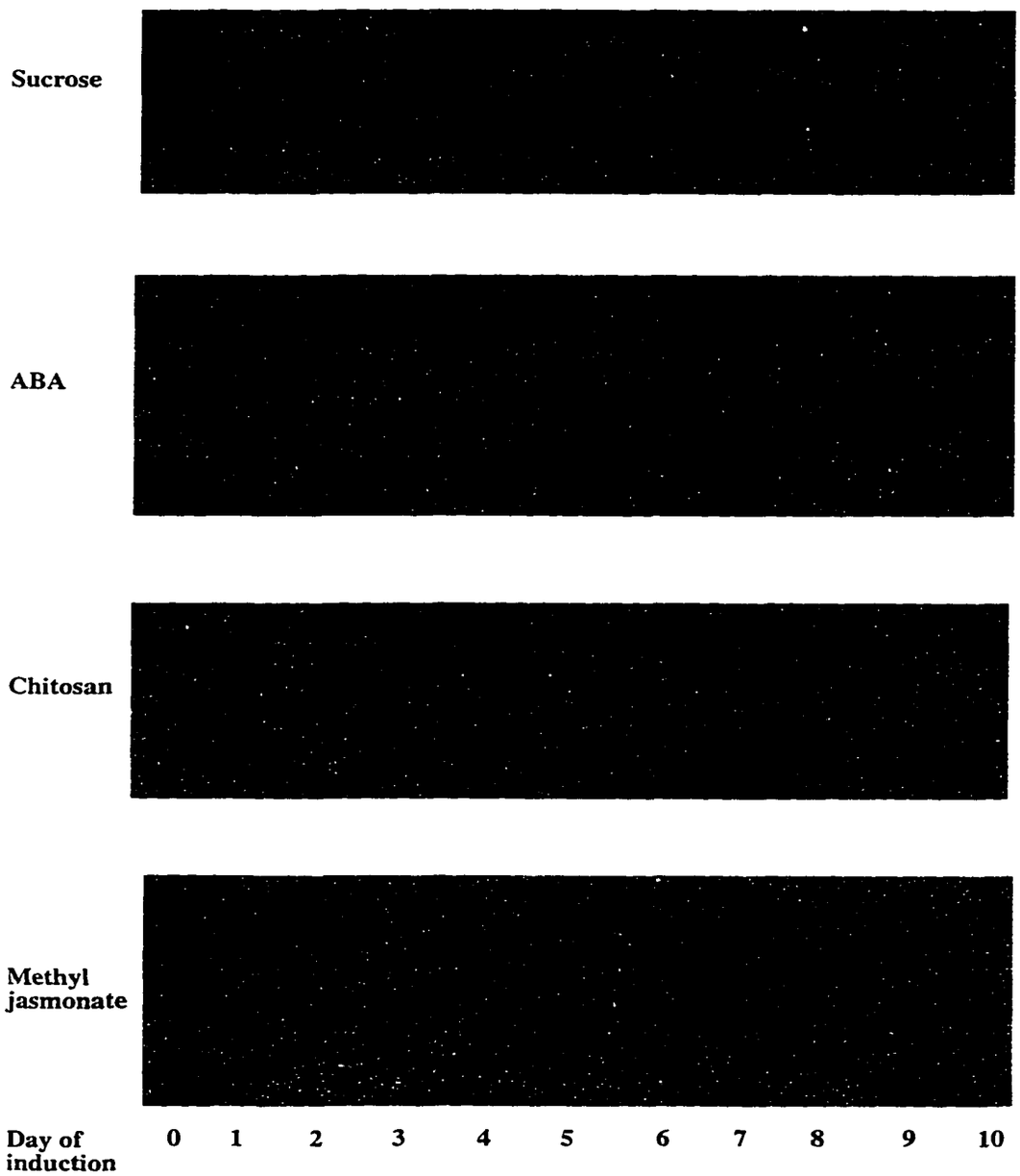
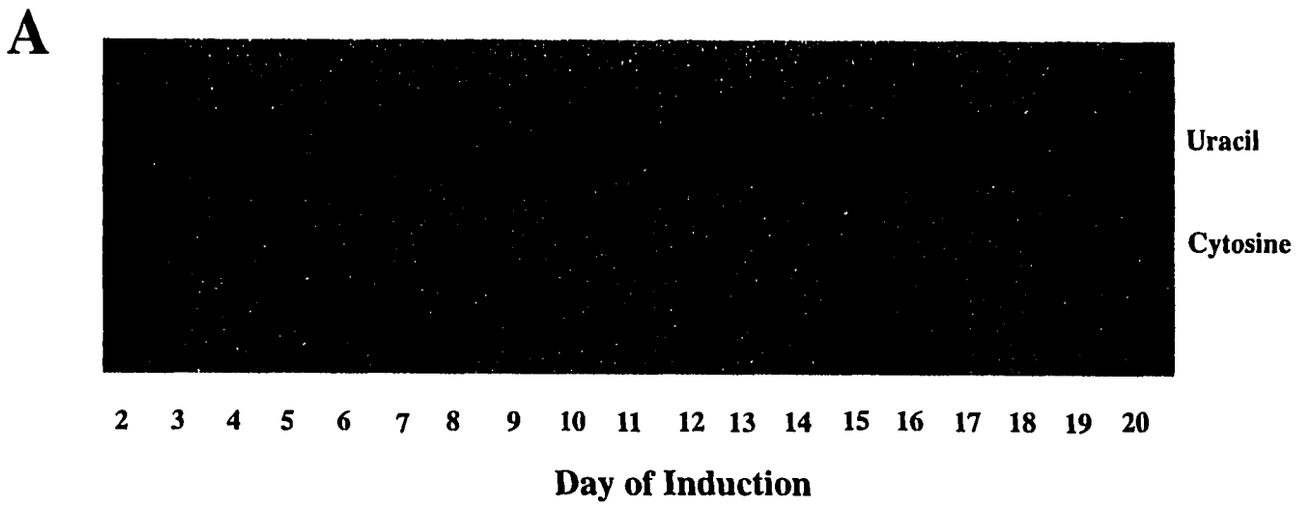

Sucrose
ABA
Chitosan
Methyl jasmonate
Day of induction
0
1
2
3
4
5
6
7
8
9
10

Figure 5


A
Uracil
Cytosine
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Day of Induction

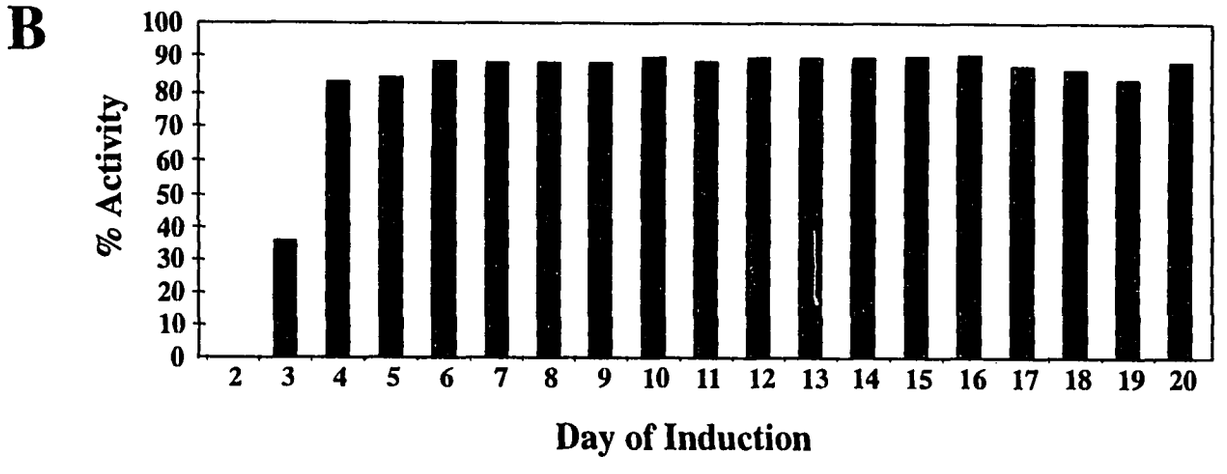

B
% Activity
100
90
80
70
60
50
40
30
20
10
0
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Day of Induction

Figure 6

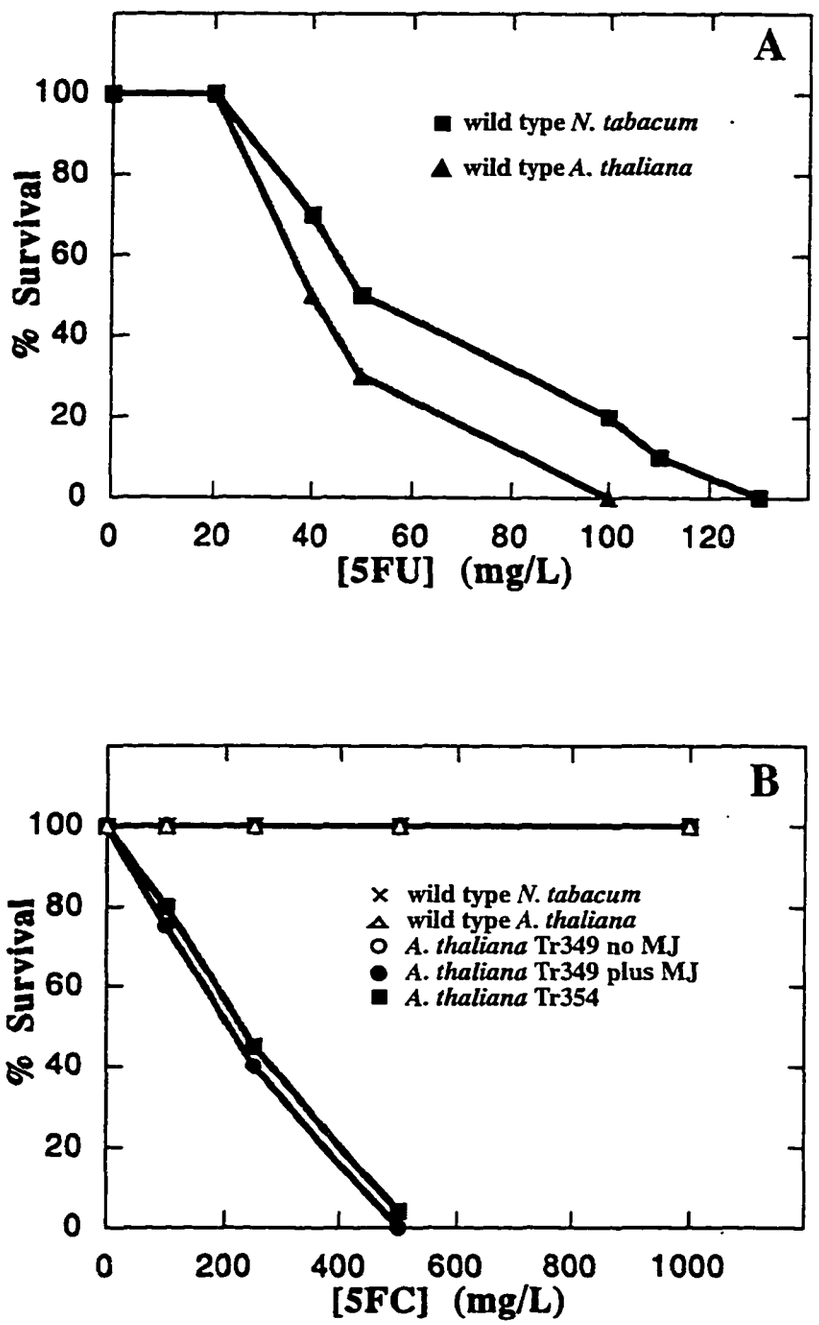

A
100
80
60
40
20
0
% Survival
0
20
40
60
80
100
120
[5FU] (mg/L)
wild type N. tabacum
wild type A. thaliana
B
100
80
60
40
20
0
% Survival
0
200
400
600
800
1000
[5FC] (mg/L)
wild type N. tabacum
wild type A. thaliana
A. thaliana Tr349 no MJ
A. thaliana Tr349 plus MJ
A. thaliana Tr354

Figure 7

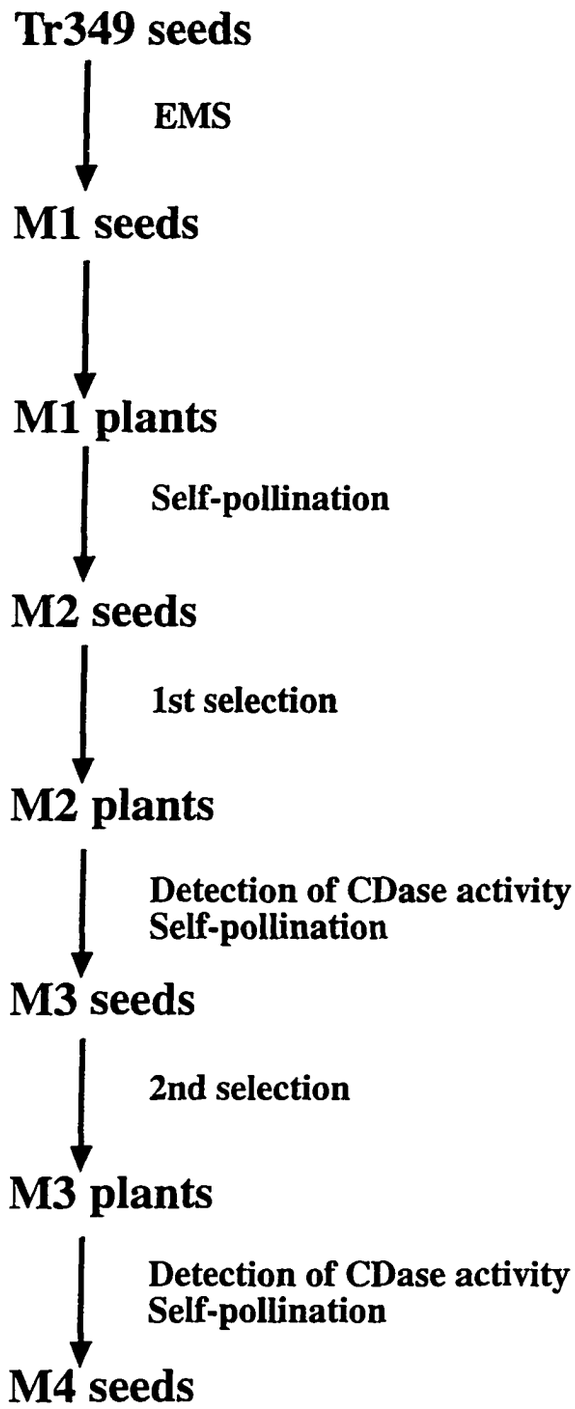

Tr349 seeds
EMS
M1 seeds
M1 plants
Self-pollination
M2 seeds
1st selection
M2 plants
Detection of CDase activity
Self-pollination
M3 seeds
2nd selection
M3 plants
Detection of CDase activity
Self-pollination
M4 seeds

Figure 8

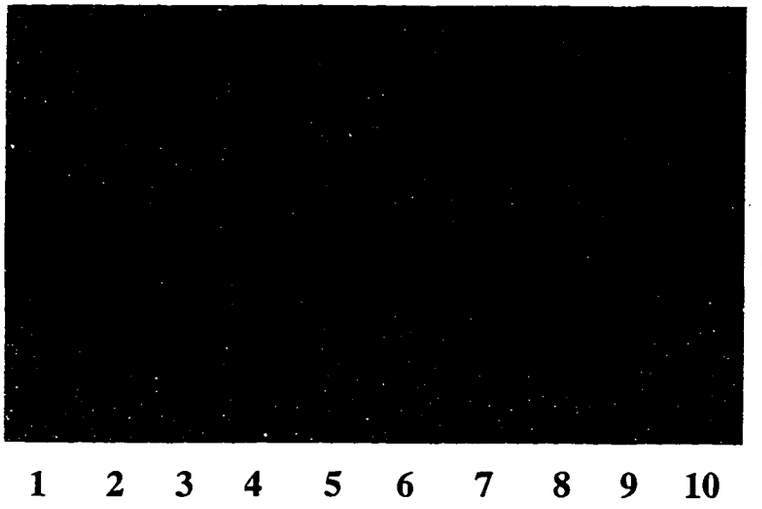


Figure 9

CHAPTER 3. ALTERATION OF NUCLEOTIDE POOLS IN TRANSGENIC PLANTS BY EXPRESSION OF *DICTYOSTELIUM DISCOIDEUM* UMP SYNTHASE IN PLANTS*

A paper to be submitted to the *Plant Journal*

Lan Zhou[1] and Robert W. Thornburg[1,2]

Abstract

We have prepared a chimeric construct containing the UMP synthase (UMPS) gene (*PYR*5-6) from *Dictyostelium discoideum* under control of the wound-inducible potato Proteinase Inhibitor II (*pin*2) promoter and have transformed *Nicotiana tabacum* cv. *Xanthi* plants with the construct. The *D. discoideum* gene contains no introns and expresses a fully functional UMP synthase protein in yeast. Southern blot analysis confirmed the presence of transgenes in the individual plants. To evaluate the expression of these transgenes, we examined UMP synthase mRNA levels by an RNase protection assay. Each of the transformed plants showed low levels of UMP synthase mRNA, which were induced by wounding. When we compared these plants for the expression of the UMP synthase activity, we found a slightly elevated level of UMP synthase expression in the unwounded state; however, the level of UMP synthase was significantly higher after induction. The results of these experiments indicated that the chimeric potato-*D. discoideum* *pin*2-*PYR*5-6 gene is functional in plants and is expressed as expected under the control of the inducible *pin*2 promoter. We also used two-dimensional, thin-layer chromatography to examine the sizes of nucleotide pools in transgenic plant leaves. These results showed that overexpression of the rate-limiting step of pyrimidine biosynthesis in plants increases the levels of UMP and UDP, but does not affect UTP, CTP or purine pool sizes.

---

*This work was sponsored by a grant (91-37301-6208) from the US Department of Agriculture.

[1]Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

[2]To whom correspondence should be addressed.

Introduction

We have previously proposed a novel method of negative selection of mutants in plants based upon the toxicity of fluoroorotic acid to plant cells (Shi and Thornburg, 1993). This scheme has long been used in yeast for selection of mutants in the pyrimidine pathway (Lacroute, 1963; Lacroute and Slonimski, 1964; Jund and Lacroute, 1970) and more recently as a tool to select against the presence of the *ura*3 and/or *ura*5 genes for genetic manipulations (Boeke, *et al.*, 1987). We have chosen to transfer this scheme into plants for the purpose of negative selection of mutants in plants.

In this scheme, the negative selection of mutants is engendered by the conversion of fluoroorotic acid into toxic metabolites. Fluoroorotic acid is taken up into plant cells and enters the pyrimidine biosynthetic pathway at the level of orotate phosphoribosyl transferase to form F-OMP. Additional catabolism by OMP decarboxylase converts F-OMP into F-UMP which is toxic. In lower eukaryotes including *Saccharomyces cerevisiae* these two enzymatic steps are encoded by separate enzymes, and selection of yeast on fluoroorotic acid results in selection of mutations in both enzymes. However, in higher eukaryotes, these two enzymatic steps have become fused into a single bifunctional protein that catalyzes the final two steps of this pathway. This bifunctional protein, termed UMP synthase, is characteristic of higher eukaryotes, including *Dictyostelium discoideum* (Jacquet *et al.*, 1988), insects (Eisenberg *et al.*, 1990), vertebrates (Suttle *et al.*, 1988) and plants (Maier *et al.*, 1995; Nasr *et al.*, 1994).

Transfer of this scheme into plants for the purpose of negative selection of mutants required the preparation of two components, plant cell lines lacking UMP synthase and an selectable UMP synthase marker gene. We have spent several years selecting plant cell lines which were deficient in UMP synthase (Santoso, 1995; Santoso and Thornburg, 1992; Santoso and Thornburg, unpublished). Due to the physical organization of both orotate phosphoribosyl transferase and OMP decarboxylase enzymatic activities on a single UMP synthase polypeptide, we had originally reasoned that selection of plant cells on fluoroorotic

acid would produce a single class of lesions which would be mutant in UMP synthase. The majority of these fluoroorotic acid resistant lines show reduced levels of UMP synthase, as expected, however, most of the analyzed resistant lines are affected in several steps in the pyrimidine biosynthetic pathway and are probably regulatory lesions (Santoso and Thornburg, submitted). In addition, one class of fluoroorotic acid resistant cell lines showed three to five-fold elevated levels of UMP synthase.

The second requirement of the reverse selection scheme is a negative selectable marker. We have therefore prepared a DNA cassette containing the UMP synthase gene (*PYR*5-6) from *Dictyostelium discoideum*. This gene contains no introns and has been used to express a functional UMP synthase that retains the ability to complement both the *ura*3 and *ura*5 mutants of *S. cerevisiae* (Shi and Thornburg, 1993). The purpose of this work is therefore two fold: first, we wish to demonstrate that the *D. discoideum* UMP synthase gene functions in plants and to evaluate the progeny of these plants expressing the foreign UMP synthase gene: and second, we wish to determine whether overexpression of the UMP synthase alone is sufficient to cause resistance to fluoroorotic acid.

In this manuscript, we express this UMP synthase cassette in transgenic tobacco plants under the control of the potato Proteinase Inhibitor II (*pin*2) promoter. The expression of this exogenous UMP synthase cassette alters UMP synthase enzymatic activity and results in modifications to pyrimidine pool sizes in whole plants.

Experimental Procedures

Materials

Nicotiana tabacum cv. *Xanthi* cell lines were maintained on a Murashige-Skoog agar medium (Murashige and Skoog, 1962) as sterile tissue cultures. Plant hormones, media components for plant tissue culture, and antibiotics were purchased from Sigma Chemical Co., St. Louis, MO. Cefotaxime (Claforan) was obtained from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ. α-[^{32}P]-UTP (800 Ci/mmol), α-[^{32}P]-dCTP (3000 Ci/mmol), carrier-

free [^{32}P]-orthophosphate (1 mCi/mL), [^{125}I]-Protein-A (70 to 100 μCi/μg), and GeneScreen™ hybridization transfer membranes were obtained from Du Pont NEN Research Products, Boston, MA. Nitrocellulose membranes for Western blot were purchased from Bio-Rad Laboratories, Hercules, CA. DNA modifying enzymes were purchased from Promega Corporation, Madison, WI. NEBlot Kit for DNA probe synthesis for Southern blot was purchased from New England Biolabs, Inc., Beverly, MA. RPA II Kit for RNase protection assay was obtained from Ambion Inc., Austin, TX. An RNA Transcription Kit for the synthesis of RNA probes was purchased from STRATAGENE, La Jolla, CA. Polyester polyethylenimine (PEI) cellulose-TLC plates were purchased from Aldrich Chemical Company, Milwaukee, WI. Other chemical reagents were from Fisher Scientific, Pittsburgh, PA. All other materials were of the highest purity available and were obtained locally.

Plant transformation and tissue culture

DNA construct pRT291 containing the wound-inducible *pin2-PYR5*-6 construct and pRT292 containing the constitutive 35S-*PYR5*-6 construct (Shi, 1991) in *E. coli* were transferred into *Agrobacterium tumefaciens* LBA4404 by triparental mating with helper pRK2073 as described (An, 1987). These *Agrobacterium* strains were used to transform leaf discs from sterile *N. tabacum* to Kanamycin resistance as previously described (Thornburg *et al.*, 1987). Transformed leaf discs were selected on a Murashige-Skoog agar medium containing 3% sucrose, kanamycin (200 mg/L), cefotaxine (500 mg/L), carbenicillin (500 mg/L), and benzylaminopurine (0.5 mg/L, for shoot induction). The co-cultivated leaf discs were incubated at 24^{0}C under light for 12 hr/day. Developing shoots were then transferred to Murashige-Skoog agar medium containing the same concentration of sucrose and kanamycin but lacking phytohormones. Regenerated plants were transferred to pots and grown in a greenhouse under natural light supplemented with artificial light (18 hr/day and 6 hr/night). The resulting transformed plants containing *pin2-PYR5*-6 were termed Tr291 plants, and those

containing 35S-*PYR5*-6 were termed Tr292 plants. Independent transformant numbers are indicated by Arabic numerals following the construct number.

DNA manipulations

DNA was isolated from the leaves of transgenic plants by the method of Dellaporta *et al.* (1983). Restricted DNA was separated by 0.7% agarose gel, denatured, neutralized, and transferred to GeneScreen™ hybridization transfer membrane, which then was hybridized according to Sambrook *et al.* (1989). DNA probes were the 1.4 Kb *Eco*RI/*Hin*dIII fragment of pRT278 labeled with α-[^{32}P]-dCTP by random primers from NEBlot Kit according to manufacturer's instructions. Probes were purified from unincorporated radioactive nucleotides on a 1 mL column of Sephadex G-75. The elute from each 200 μL fraction was loaded onto a PEI cellulose-TLC plate. The plate was developed in 0.75 M KH_2PO_4, pH 3.4 and exposed to an X-ray film for 5 min. Fractions showing the highest level of incorporated radioactivity were combined and used for hybridization.

*pin*2 induction and mRNA isolation

The leaves from greenhouse-grown plants were cut into two halves at the midvein. Half of the leaf blade, without the midvein, was removed with a razor blade and frozen at -70^{0}C. The remaining leaf blade with the attached midvein was placed in Murashige-Skoog liquid medium containing 3% sucrose. After an 18 to 24 hr incubation, the remaining leaf blade was removed from the midvein and frozen. Subsequently, both leaf blade halves were processed simultaneously. Total RNAs were extracted according to the method of Wadsworth *et al.* (1988).

RNase protection assay (RPA)

The probe used to detect *D. discoideum* UMP synthase mRNA in Tr291 leaves by RPA was derived from pRT345. The plasmid, pRT345, was made by inserting the 333 bp *Hin*dIII/*Kpn*I fragment of *D. discoideum* UMPS fragment from pRT278 (Shi and Thornburg, 1993) into the vector pBluescript II SK+ to form the plasmid pRT345. A 341 nucleotide

labeled antisense probe was made by linearizing pRT345 with *Hin*dIII and transcribing RNA with T7 RNA polymerase. The DNA template was removed with RNase-free DNase I (Ambion) at 37°C for 30 min. The probes were purified in a 5% polyacrylamide-8M urea gel. The gel was exposed to X-ray film at room temperature for 5 min to allow detection of the full-length in vitro transcript. The full-length band was excised and then eluted at 37°C for 4 hr in 350 µL of elution buffer. The eluted probes were sometimes stored at -20°C for several days.

A control sense strand UMP synthase RNA was synthesized from *Hin*dIII-linearized pRT342 by T3 RNA polymerase. The plasmid, pRT342, was made by inserting the *Pst*I/*Eco*RI fragment of pRT278 into the vector pBluescript II SK+.

RNase Protection Assays were conducted according to the method of Higgs and Colbert (1992). The 341 nucleotide gel purified RNA probe (1×10^4 to 1×10^5 cpm) was added to 50 µg of total RNA from Tr291 leaves or 500 pg of full-length in vitro transcribed UMP synthase sense-strand RNA in the total volume of 25 µL. The probe and sample RNAs were coprecipitated and hybridized in 20 µL of hybridization buffer for 14 hr. at 45°C. Single-stranded RNAs were digested by 0.5 units/mL RNase A and 100 units/mL RNase T_1. The RNAs were pelleted, dissolved in 8 µL gel loading buffer, heated for 3 to 4 min at 90°C, and loaded on a 5% polyacrylamide-8M urea gel. A ladder of [^{32}P]-labeled, *Afl*III digested bacteriophage lambda DNA was used to mark molecular weight. After electrophoresis, the gel was transferred onto a piece of filter paper, covered with plastic wrap, and exposed to X-ray film at -70°C with an intensifying screen.

UMP synthase assay

The leaves of greenhouse-grown Tr291 plants were used to measure UMP synthase as described (Santoso and Thornburg, 1992). The leaves were harvested and induced by 3% sucrose for 18 to 24 hr (Thornburg *et al.*, 1990). After induction, the leaves were

homogenized and the protein content of the leaves was determined (Bradford, 1976). Aliquots containing 1 mg of total protein were assayed for UMP synthase activity.

Nucleotide analysis

Leaves (0.5 g) from Tr291#1 plants were cut into 1 cm squares and labeled with carrier-free [^{32}P]-orthophosphate (final activity 100 μCi/mL) at room temperature for 3 to 4 hr to achieve a steady state level of labeling (Raz and Fluhr, 1993). The labeled leaf squares were transferred to 3% sucrose solution for induction. Uninduced controls were incubated for the same time without sucrose. The conditions of induction were at room temperature for 18 to 24 hr. Leaf squares were washed twice with water. Labeled cellular nucleotides were extracted by homogenizing leaf squares in 500 μL of ice-cold 2 M formic acid, and the extracts were incubated on ice for 30 min. To selectively precipitate unincorporated inorganic phosphate, one tenth volume of a freshly prepared solution containing 400 mM sodium tungstate, 500 mM tetraethylammonium·HCl, and 500 mM procaine·HCl in the ratio 5:4:1 (v/v/v) was added to the extracts (Bochner and Ames, 1982).

A mixture of 1 to 2 μL of nucleotide extracts (1.0 X 10^5 to 1.0 X 10^6 cpm) and 50 nmoles of unlabelled pure nucleotides was loaded onto a PEI cellulose-TLC plate. The plate was immersed in methanol for 5 min to remove interfering salts and to dehydrate the layers as described by Randerath and Randerath (1964). Nucleotides labeled with [^{32}P] were separated by two dimensional TLC. The solvent system for UMP described by Liljelund and Lacroute (1986) was used. UDP pools were determined by using the solvent system of Ginther and Ingraham (1974). The solvent system for the determination of NTP pools was that described by Neuhard *et al.* (1965). After the first dimensional development, the plate was dried and immersed in 0.01 M Tris in methanol for 5 min and then in methanol alone for 15 min (Randerath and Randerath, 1965). Following the development of the second dimension, the plate was air dried for 10 to 15 min, wrapped in plastic wrap, and exposed to X-ray film at -70°C.

Results

Plant transformation and molecular analysis of transgenic plants

To establish Tr291 transgenic plants, we moved the pRT291 construct into *A. tumefaciens* LBA4404 and subsequently transformed *N. tabacum* to kanamycin resistance. New plantlets were regenerated from the leaf discs after about 1 month and were transferred to jars for further incubation. The plants were transferred to soil in pots and grown in a greenhouse. When the plants were 10 to 15 cm tall, after about 2 months, analysis of these plants began.

To determine that the kanamycin-resistant plants were indeed transgenic, we assayed for the presence of the transgene by Southern blot analysis. The results (Figure 1, Panel A) show the presence of transgenes in the individual Tr291 plants. Plant DNA was digested with *Xba*I, which cuts within the construct at each end of the UMP synthase gene, or *Hin*dIII, which cuts only once within the construct. Therefore the number of bands present in these *Hin*dIII digests shows the number of the copies of transgenes in the genome of that plant. The *D. discoideum* UMP synthase gene isolated from *Xba*I digestion of pRT286 (Shi and Thornburg, 1993) was used to mark the size of the UMP synthase gene. Untransformed *N. tabacum* does not contain a sequence hybridizing to the *D. discoideum* UMP synthase (*PYR5-6*) gene, as evidenced by the lack of bands in both *Xba*I and *Hin*dIII digestion. Plants Tr291#1 and Tr291#2 each contained two copies of the construct. The sizes of the bands are 2.8 Kb and 3.5 Kb for Tr291#1 and 11.6 Kb and 23.0 Kb for Tr291#2. In both cases, however, when digested with *Xba*I, a single band corresponding to the size of the UMP synthase gene resulted. Other transformed plants produced in this study contained either one or two copies of the construct. Because we observed the presence of UMP synthase hybridizing bands in the kanamycin resistant plants, we concluded that these plants are transgenic, containing either one or two copies of the transgene.

Because these plants were demonstrated to be transgenic, we wanted to determine whether the genes were expressed. We therefore used RNase protection assays to examine the expression of the *D. discoideum* UMP synthase mRNA driven by the *pin*2 promoter. To perform these analyses, half of the leaf was removed as an uninduced control and frozen at -70^{0}C. The second half was induced as described in experimental procedures. After induction, the second half of the leaf was also frozen at -70^{0}C. Both leaf halves were subsequently assayed together. As shown in Figure 1, Panel B, both Tr291#1 and Tr291#2 plants showed a low, constitutive level of UMP synthase mRNA, which could be induced several fold by wounding. The other transgenic plants showed a similar result. Untransformed *N. tabacum* leaves were induced as described and used as a negative control; mRNA isolated from these leaves showed no detectable signal. Thus, in the uninduced state, all the transgenic plants gave weak signals, indicating that there was some leaky expression of the *pin*2-*PYR*5-6 construct.

Most of the radiolabeled signal detected in these assays was in the form of the full-sized 341 bp transcript, but some smaller radioactive material was occasionally observed. These smaller sized products may be due to incomplete digestion during the RNase treatment or radiolytic decay of the probe. The RNase protection assay results demonstrated that the *pin*2-*PYR*5-6 construct in transgenic plants produced a UMP synthase hybridizing transcript of the size expected and in a wound-inducible manner.

Assay of UMP synthase and nucleotide analysis

Because the RNase protection assays results demonstrated that *D. discoideum* UMP synthase mRNA was expressed in plants, we decided to examine whether the plants expressing the *pin*2-*PYR*5-6 construct had a higher level of enzyme activity than control plants. When we examined these plants for the expression of the UMP synthase activity, we observed a slightly elevated level of UMP synthase expression in the uninduced state. This was expected, because of the previously observed leaky expression of the construct detected by the RNase protection assay. However, the level in the induced state is much higher for Tr291#1 and Tr291#2 plants

than in the uninduced state. If we subtract background levels provided by the plant UMP synthase, we see that the level of induction is 2- to 4-fold greater than that in the uninduced state. Thus the *D. discoideum* UMP synthase expression cassette gene functions in plants as it does in yeast to produce an active UMP synthase enzyme.

The plants produced in this study express a unique UMP synthase gene under the control of the wound-inducible *pin*2 promoter. As previously stated, UMP synthase is the rate-limiting step in the synthesis of pyrimidine nucleotides. Because of this uniquely expressed UMP synthase gene, we wanted to determine whether this genotype conferred any phenotype upon the plants. In the greenhouse, no physical difference was observed between the transformants and the untransformed plants. We therefore decided to determine whether there was a biochemical phenotype associated with the expression of the UMP synthase cassette. To examine the biochemical phenotype of these plants, we examined the levels of pyrimidine nucleotides in these plants. Because of the inducible nature of the *pin*2 promoter, we chose to compare the transgenic plant tissues from single plants in both the uninduced and induced states.

We used two-dimensional, thin-layer chromatography to examine the sizes of nucleotide pools in transgenic plant leaves to determine whether overexpression of the rate-limiting step of pyrimidine biosynthesis in plants alters nucleotide pools. The nucleotide pools in transgenic plant leaves were labeled with [^{32}P]-orthophosphate and separated by 2D-PEI cellulose TLC plates as described in Experimental Procedures. Results from a typical set of analysis are shown in Figure 3. After TLC analysis and exposure to X-ray film, the location of nucleotides were determined by the position of the unlabelled nucleotides under short-wave ultraviolet light. The position of the labeled spot was determined by autoradiography. The autoradiogram was used to locate radioactive spots on the chromatogram. To determine the amount of radioactivity in a given spot, the spot was circled with a soft lead pencil, cut out with scissors, placed in a vial containing scintillation fluid, and counted in a scintillation counter.

The absolute size of the individual nucleotide pools was calculated from the radioactivity present in each spot on the chromatogram and the specific activity of [^{32}P]-phosphate. The results from a series of similar assays performed on Tr291#1 are presented in Table 1. As expected, these data show that the pyrimidine pool sizes are modified by induction of the *pin*2-*PYR*5-6 construct. The primary difference was observed in the UMP pool, where the pool size doubled after induction. Because UMP is the product of the overexpressed UMP synthase, this result was expected. In addition to the elevated UMP pool, the induced tissues also showed elevated levels of the UDP pool. However, the elevation of the UDP pool (1.3-fold) is much lower than the elevation observed for the UMP pool (2.1-fold). Whether the lower level of the UDP pool is caused by the inefficiency of UMP kinase or feedback inhibition by the saturated UTP pool is not clear. The nucleotide triphosphate pools are not significantly different between the uninduced and induced tissues. Thus, overexpression of UMP synthase did not affect the overall nucleotide triphosphate pools in our transgenic plants.

Resistance to 5-FOA in Tr292 plants

Our laboratory has produced a large number of fluoroorotic acid resistant callus lines (Santoso and Thornburg, unpublished). Of these lines, one phenotypic class, which represents 14% of total isolates, shows the up-regulation of several numbers of the pyrimidine biosynthetic pathway, including ATCase, UMP synthase, thymidylate synthase, and perhaps other enzymes (Santoso and Thornburg, unpublished). Because UMP synthase is the rate-limiting step of UMP synthesis, the plants produced in these studies permit us an opportunity to test whether overexpression of UMP synthase is sufficient to lead to fluoroorotic acid resistance. The Tr292 plants expressing the 35S-*PYR*5-6 construct were prepared with the same methods as the Tr291 plants. These plants constitutively expressed UMP synthase at levels between 2-fold and 5-fold higher than the wild-type plants. To test whether the overexpression of UMP synthase levels results in resistance to 5-fluoroorotic acid, F1 seeds of Tr292 transgenic plants were grown on MS media containing kanamycin to insure the presence

of the linked 35S-*PYR*5-6 gene. Then the plantlets containing transgenes were transplanted onto MS media containing 5-fluoroorotic acid at a density of 40 plantlets per plate. In total, 4 plates were used for this experiment. After about 2 weeks on media containing 5-fluoroorotic acid, the plantlets began to turn yellow and eventually all plants died. These results demonstrate that overexpression of UMP synthase alone is not sufficient to cause resistance to 5-fluoroorotic acid.

Discussion

We have linked a UMP synthase gene (*PYR*5-6) from *D. discoideum* to a potato wound-inducible Proteinase Inhibitor II (*pin*2) promoter. To evaluate the function of this UMP synthase gene of *D. discoideum* in plants, we have transformed the construct *pin*2-*PYR*5-6 into *N. tabacum* and regenerated transgenic plants. The presence of the transgene was verified by Southern blot analysis. The inducible expression of the construct was confirmed by RNase protection assays and by enzyme assays. All the transgenic plants showed the wound-inducible overexpression of UMP synthase.

To examine whether there was a biochemical phenotype associated with the overexpression of the UMP synthase, we examined the sizes of nucleotide pools in both uninduced and induced leaves of transgenic plants. In these studies, we found that UMP and UDP pool sizes were elevated in the induced state relative to the uninduced leaves. However UTP and CTP pool sizes were not significantly different in induced and uninduced leaves. Purine pool sizes were not affected.

We have produced many mutant lines of *N. plumbaginifolia* callus that can grow on 5-FOA. One group of calli, which represents 14% of our isolates has elevated levels of UMP synthase (>300% of wild-type), as well as of other pyrimidine biosynthetic enzymes. The plants prepared for this study permitted us to determine whether overexpression of UMP synthase alone was responsible for the 5-FOA resistant phenotype. To address this question, we evaluated our Tr292 plants which were transformed with 35S-*PYR*5-6 for growth in the

presence of 5-FOA. These plants show high levels of UMP synthase from 200% to nearly 500% of wild-type expression. We therefore tested these Tr292 plants for resistance to 5-FOA. After approximately 2 weeks on 5-FOA, the plantlets of Tr292 F1 seeds began to turn yellow and die. Thus, the overexpression of UMP synthase alone is not sufficient to cause resistance to 5-FOA. Therefore, resistance to 5-FOA requires more than just the elevated levels of UMP synthase, perhaps the elevated levels of TS or ATCase or another step not elucidated.

Finally, our intent was to demonstrate that the *PYR*5-6 cassette prepared from *D. discoideum* could function in plants as it does in yeast to produce a fully functional UMP synthase enzyme. Based upon these results, we conclude that it does. The expression of *PYR*5-6 has been previously used as the basis of a powerful reverse selection scheme in *S. cerevisiae* (Boeke *et al.*, 1984), *Aspergillus flavus* (Woloshuk *et al.*, 1989), *D. discoideum* (Kalpaxis *et al.*, 1991), and *N. tabacum* (Santoso and Thornburg, 1992). This selection scheme selects for mutants that fail to express *PYR*5-6. By expressing this *PYR*5-6 cassette under the control of the *pin*2 promoter, we hope to be able to select for mutations that block activation of the inducible promoter.

Because the *D. discoideum* probe does not hybridize to sequences in *N. tabacum*, and also because antiserum raised against the plant UMP synthase, or against the yeast ODCase, does not recognize the *D. discoideum* UMP synthase protein, we concluded that the *D. discoideum* UMP synthase is unique from plant UMP synthase. Thus, *D. discoideum* UMP synthase is a good marker for use in plant systems because it is significantly different at both the gene and protein levels and the probes of plant UMP synthase do not recognize either the *D. discoideum* gene or protein.

References

An, G. (1987) Binary vectors. Methods Enzymol. 153, 292-305.

Bochner, B.R. and Ames, B.N. (1982) Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. J. Biol. Chem. 257, 9759-9769.

Boeke, J.D., Lacroute, F. and Fink, G.R. (1984) A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoroorotic acid resistance. Mol. Gen. Genet. 197, 345-346.

Boeke, J.D., Trueheart, J., Natsoulis, G. and Fink, G.R. (1987) 5-fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. 154, 163-175.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72, 248-254.

Dellaporta, S., Wood, J. and Hicks, J.B. (1983) A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1, 19-21.

Eisenberg, M., Gathy, K., Vincent, T. and Rawls, J. (1990) Molecular cloning of the UMP synthase gene rudimentary-like from *Drosophila melanogaster*. Mol. Gen. Genet. 222, 1-8.

Ginther, C.L. and Ingraham, J.L. (1974) Cold-sensitive mutant of *Salmonella typhimurium* defective in nucleoside diphosphokinase. J. Bacteriol. 118, 1020-1026.

Higgs, D.C. and Colbert, J.T. (1992) RNase protection assays and RNA gel blots: a direct comparison of sensitivity. Genetic Analysis: Techniques and Applications 9, 146-148.

Jacquet, M., Guilbaud, R. and Garreau, H. (1988) Sequence analysis of the *DdPYR*5-6 gene coding for UMP synthase in *Dictyostelium discoideum* and comparison with orotate phosphoribosyl transferases and OMP decarboxylases. Mol. Gen. Genet. 211, 441-445.

Jund, R. and Lacroute, F. (1970) Genetic and physiological aspects of resistance to 5-fluoropyrimidines in *Saccharomyces cerevisiae*. J. Bacteriol. 102, 607-615.

Kalpaxis, D., Zundorf, I., Werner, H., Reindl, N., Boy-Marcotte, E., Jacquet, M. and Dingermann, T. (1991) Positive selection for *Dictyostelium discoideum* mutants lacking UMP synthase activity based on resistance to 5-fluoroorotic acid. Mol. Gen. Genet. 225, 492-500.

Lacroute, F. (1963) Génétique de la résistance au 5-fluorouracile chez la levure. C.R. Acad. Sci. 257, 4213-4216.

Lacroute, F. and Slonimski, P.P. (1964) Etude physiologique des mutants résistants au 5-fluorouracile chez la levure. C.R. Acad. Sci. 258, 2172-2174.

Liljelund, P. and Lacroute, F. (1986) Genetic characterization and isolation of the *Saccharomyces cerevisiae* gene coding for urine monophosphokinase. Mol. Gen. Genet. 205, 74-81.

Maier, T., Zhou, L. and Thornburg, R.W. (1995) Nucleotide sequence of a cDNA encoding UMP synthase from *Nicotiana tabacum* (GenBank U22260). Plant Physiol. 108, 1747.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473-497.

Nasr, F., Berthauche, N., Dufour, M.E., Minet, M. and Lacroute, F. (1994) Heterospecific cloning of *Arabidopsis thaliana* cDNAs by direct complementation of pyrimidine auxotrophic mutants of *Saccharomyces cerevisiae*. I. Cloning and sequence analysis of two cDNAs catalyzing the second, fifth and sixth steps of the *de novo* pyrimidine biosynthesis pathway. Mol. Gen. Genet. 244, 23-32.

Neuhard, J., Randerath, E. and Randerath, K. (1965) Ion-exchange thin-layer chromatography XIII. Resolution of complex nucleoside triphosphate mixtures. Anal. Biochem. 13, 211-222.

Randerath, K. and Randerath, E. (1964) Ion-exchange chromatography of nucleotides on poly-(ethyleneimine)-cellulose thin layers. J. of Chromatography 16, 111-125.

Randerath, K. and Randerath, E. (1965) Ion-exchange thin-layer chromatography XIV. Separation of nucleotide sugars and nucleoside monophosphates on PEI-cellulose. Anal. Biochem. 13, 575-579.

Raz, V. and Fluhr, R. (1993) Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell 5, 523-530.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Santoso, D. (1995) 5-Fluoroorotic acid-selected cell lines and regulation of UMP synthase gene expression in tobacco cells. Ph.D. thesis. Iowa State University, Ames.

Santoso, D. and Thornburg, R.W. (1992) Isolation and characterization of UMP synthase mutants from haploid cell suspensions of *Nicotiana tabacum*. Plant Physiol. 99, 1216-1225.

Shi, N.Q. (1991) Construction of a UMP synthase gene for use as a negative selectable marker in plants. M.S. thesis Iowa State University, Ames.

Shi, N.Q. and Thornburg, R.W. (1993) Construction of a UMP synthase expression cassette from Dictyostelium discoideum. Gene 127, 199-202.

Suttle, D.P., Bugg, B.Y., Winkler, J.K. and Kanalas, J.J. (1988) Molecular cloning and nucleotide sequence for the complete coding region of human UMP synthase. Proc. Natl. Acad. Sci. USA 85, 1754-1758.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R. and Ryan, C.A. (1987) Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyl transferase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA 84, 744-748.

Thornburg, R.W., Andrea, K. and Molin, L. (1990) Chloramphenicol acetyl transferase (CAT) protein is expressed in transgenic tobacco in field tests following attack by insects. Plant Physiol. 92, 500-505.

Wadsworth, G.J., Redinbaugh, M.G. and Scandalios, J.G. (1988) A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. Anal. Biochem. 172, 279-283.

Woloshuk, C.P., Seip, E.R., Payne, G.A. and Adkins, C.R. (1989) Genetic transformation system for aflatoxin-producing fungus, *Aspergillus flavus*. Appl. Environ. Micro. 55, 86-90.

Table 1. Nucleotide pool sizes in uninduced and induced Tr291#1 plants

	N	Average ± Std Dev (x 10^{-6})[a] Uninduced (U)	Induced (I)	I/U
UMP	4	698 ± 57	1,495 ± 118	2.1[b]
UDP	5	228 ± 11	288 ± 13	1.3[b]
UTP	9	32 ± 13	22 ± 8	0.7[c]
CTP	9	19 ± 6	15 ± 11	0.8[c]
ATP	5	238 ± 16	228 ± 16	1.0[c]
GTP	9	17 ± 5	16 ± 5	0.9[c]

[a]Pool levels are expressed as absolute pool sizes (pmoles per 1 milligram of leaf). Std Dev: sample standard deviation. N: the number of assays for each nucleotide.

[b]Analysis by Student's *t* test indicates that these values for the uninduced and induced tissues are significantly different at the 98% confidence level.

[c]Not significantly different by Student's *t* test at the 98% confidence level.

Figure Legends

Figure 1. Molecular analysis of Tr291#1 and Tr291#2 plants.

Panel A, Southern blot. C: positive control, 50 ng *D. discoideum* UMP synthase gene isolated from *Xba*I digestion of pRT286. *Nt*: wild-type *N. tabacum*. H: *Hin*dIII digestion. X: *Xba*I digestion. 10 µg of leaf DNA was loaded per lane. **Panel B,** Expression of the *pin*2-*PYR*5-6 construct in the transformed Tr291#1 and Tr291#2 plants measured by RNase protection assay. A sense strand UMP synthase RNA was used to mark the 0.34 Kb band (not shown). *Nt*: wild-type *N. tabacum* control; U: uninduced; I: induced; 50 µg of total RNA of tobacco leaf was assayed per lane.

Figure 2. UMP synthase activity in the uninduced and induced Tr291#1 and Tr291#2 plants.

Tr307#6: *N. tabacum* negative control plant. U: uninduced; I: induced. 1 mg of total protein of tobacco leaf was assayed for UMP synthase activity. These results are the average of three repetitions for each plant. Standard deviation is given by the error bars.

Figure 3. Autoradiograms of two-dimensional separation of nucleotides in the uninduced and induced Tr291#1 plants on polyethyleneimine (PEI)-cellulose TLC.

Leaf discs were labeled by carrier-free [^{32}P] orthophosphate for 22 hr, then induced by 3% sucrose for 18 to 24 hr. Cellular nucleotides were extracted by 2N formic acid. 1 X 10^5 and 1 X 10^6 cpm of extract was loaded onto a TLC plate for UMP or UDP pool and NTP pools, respectively as described in Experimental Procedures. **Panel A and B:** UMP pools; **Panel C and D:** UDP pools; **Panel E and F:** NTP pools. **Panel A, C, E:** uninduced; **Panel B, D, F:** induced.

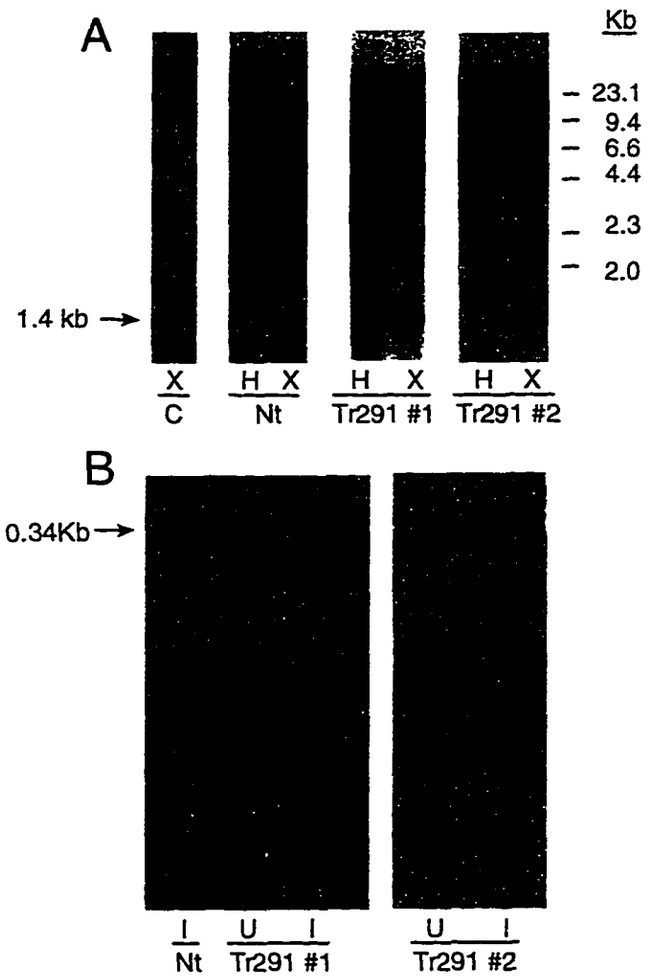

A
Kb
23.1
9.4
6.6
4.4
2.3
2.0
1.4 kb
X
C
H X
Nt
H X
Tr291 #1
H X
Tr291 #2
B
0.34Kb
I
Nt
U I
Tr291 #1
U I
Tr291 #2

Figure 1

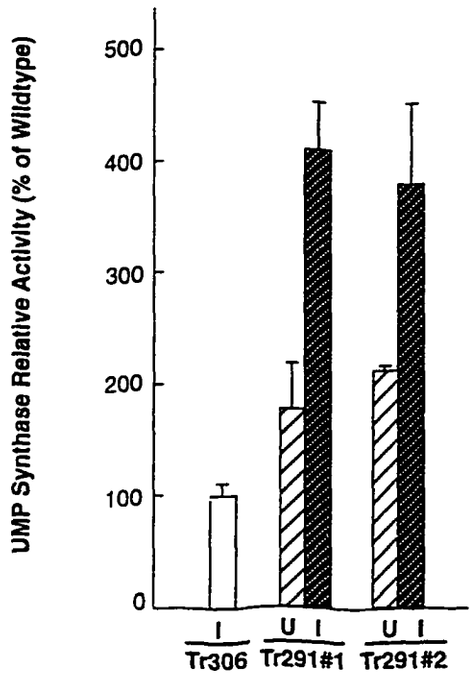

UMP Synthase Relative Activity (% of Wildtype)
500
400
300
200
100
0
I
U I
U I
Tr306
Tr291#1
Tr291#2

Figure 2

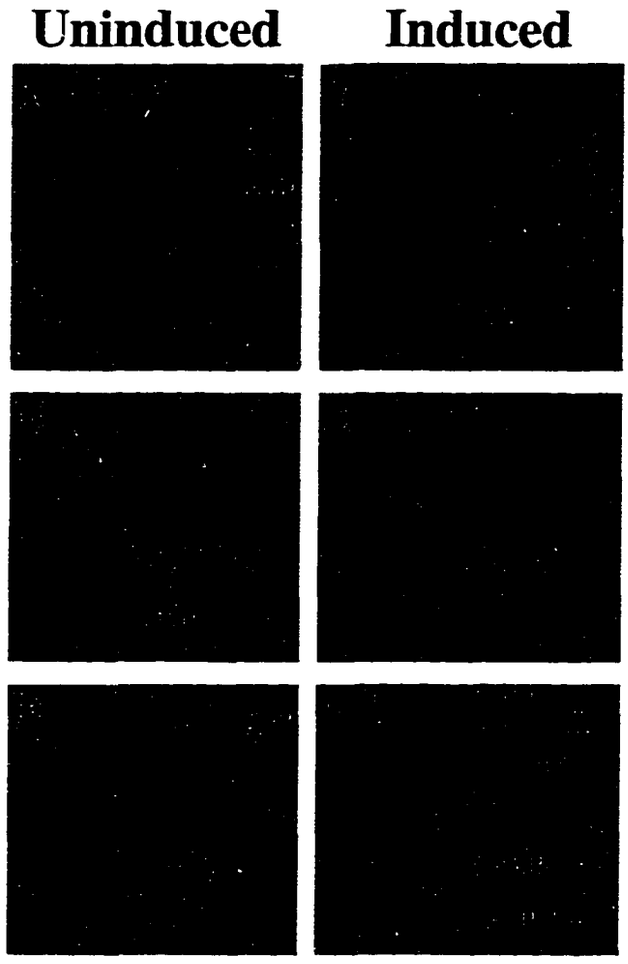


Figure 3

CHAPTER 4. CLONING, EXPRESSION IN *E. COLI* AND CHARACTERIZATION OF *ARABIDOPSIS THALIANA* UMP/CMP KINASE*

A paper accepted by the *Plant Physiology*

Lan Zhou[1], François Lacroute[2] and Robert W. Thornburg[1,3]

Abstract

A cDNA encoding the *Arabidopsis thaliana* UMP/CMP kinase was isolated by complementation of a *Saccharomyces cerevisiae ura*6 mutant. The deduced amino acid sequence of the plant UMP/CMP kinase showed 50% identity with other eukaryotic UMP/CMP kinase proteins. The cDNA was subcloned into pGEX-4T-3 and expressed as a GST-fusion protein in *E. coli*. Following proteolytic digestion, the plant UMP/CMP kinase was purified and analyzed for its structural and kinetic properties. The mass, N-terminal sequence and total amino acid composition agreed with the sequence and composition predicted from the cDNA sequence. Analytical ultracentrifugation revealed that the protein exists as a dimer. Kinetic analysis revealed that the UMP/CMP kinase preferentially uses ATP (K_m = 29 μM when UMP is the other substrate and K_m = 292 μM when CMP is the other substrate) as a phosphate donor; however, both UMP (K_m = 153 μM) and CMP (K_m = 266 μM) were equally acceptable as the phosphate acceptor. The optimal pH for the enzyme is 6.5. Inhibition studies using Ap_5A indicate that Ap_5A is a competitive inhibitor of both ATP and UMP.

---

*This work was sponsored by a grant (91-37301-6208) from the US Department of Agriculture. Journal paper number J-17416 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3340.

[1]Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

[2]Centre de Génétique Moléculaire, Centre Nationale de La Recherche Scientifique, 22 Avenue de la Terrace, 91198 Gif sur Yvette, Cedex France.

[3]To whom correspondence should be addressed.

Introduction

Pyrimidines are intimately involved in the normal physiology of cells. They participate at multiple levels in intermediary and secondary metabolism from nucleotide and macromolecule biosynthesis, to the biosynthesis of complex carbohydrates, to the metabolic regulation of intermediary metabolism. All pyrimidines within the cell are derived from UMP. UMP arises either from the *de novo* pyrimidine biosynthetic pathway or from salvage pathways. UMP/CMP kinase converts uridine and cytidine monophosphates (UMP and CMP) into the corresponding uridine diphosphate (UDP and CDP). Because all pyrimidines are derived from UMP, UMP kinase is the first committed step and one of the central enzymes in the further anabolism of pyrimidine nucleotides.

The importance of pyrimidine monophosphokinases to cell physiology has been firmly established. In both bacteria and in yeast, the roles of pyrimidine monophosphokinases with respect to cell proliferation and physiology has been widely studied. In *E. coli*, the UMP kinase (*pyrH/smbA*) gene product has been shown to influence cell proliferation. Yamanaka *et al.*, (1992), working with the *mukB* gene, have isolated a suppressor of *mukB* that they term *smbA*. The phenotype described for the *smbA* phenotype is pleiotrophic. First, the *smbA* mutant ceased macromolecular synthesis, was hypersensitive to SDS, and showed a novel morphological phenotype under nonpermissive conditions. Later, it was found that the wild-type *smbA* gene was identical to the *pyrH* gene and that the *smbA2* mutant protein encodes an unstable UMP kinase with impaired catalytic and regulatory functions. Complementation of this mutant permitted the first isolation of a eukaryotic UMP kinase gene (Liljelund and Lacroute, 1986). This is the same mutant that we have complemented in this study to isolate the *Arabidopsis thaliana* UMP/CMP kinase cDNA.

In yeast, mutations at the UMP kinase gene have been shown to cause a conditional lethal phenotype (Liljelund and Lacroute, 1986). When grown at the nonpermissive

temperature, the UTP and CTP pools decline to 10% of their wild-type levels affecting both RNA and protein synthesis, which ultimately results in cell death.

Other pyrimidine monophosphokinases are also important in normal cellular physiology. The *cdc8* mutant of *S. cerevisiae* was isolated as a cell cycle deficient mutant that was defective in nuclear division (Newlon and Fangman, 1975). When incubated at the restrictive temperature, the *cdc8* mutants arrest in S phase with a typical dumbbell morphology. This mutation also has pleiotrophic effects other than morphology, including inhibition of normal cellular DNA replication (Birkenmeyer et al., 1984) involvement in error-prone repair (Prakash et al., 1979; Baranowska and Zuk, 1991); and involvement in repair of single-stranded breaks (Baranowska et al., 1990). The *CDC8* gene was isolated (Birkenmeyer et al., 1984; Kuo and Campbell, 1991), and later, it was determined that this mutation encodes dTMP kinase (Jong et al., 1985). Known suppressors of the *CDC8* gene have also been isolated and characterized. One of these, *SOC8,* encodes the UMP kinase (*ura*6) gene (Jong et al., 1993). Thus, nucleotide monophosphokinases can have profound effects on cellular morphology and physiology.

The substrate utilization by the eukaryotic UMP kinases has also received much study (Jong et al., 1993; Weismüller et al., 1990; Müller-Dieckmann and Schultz, 1994). Whereas all eukaryotic enzymes have specificity for both UMP and CMP, the yeast enzyme also has specificity for AMP. Indeed, the yeast UMP kinase has such a high affinity for AMP that the *ura*6 gene has been isolated as multicopy suppressors of yeast deficient in adenylate kinase (Schricker et al., 1992). The yeast uridine kinase gene also complements the *E. coli* adenylate kinase enzyme. In contrast the *D. discoideum* enzyme does not complement either the yeast or the *E. coli* adenylate kinase mutants. UMP kinase is also required in the metabolic activation of several important antitumor drugs including 5-fluorouracil and Ara-C (Seagrave and Reyes, 1987); consequently, UMP kinase plays a central and very important role in pyrimidine anabolism.

In addition to studies of the eukaryotic enzyme, the prokaryotic enzyme has also received much attention (Yamanaka et al., 1992; Valentin-Hansen, 1978; Serina et al., 1995, 1996). Because of this, significant differences between the enzymatic activities of the prokaryotic and eukaryotic enzymes have been identified. The prokaryotic enzyme is allosterically regulated by both GTP and by UTP (Serina et al., 1995). GTP functions to stimulate the enzyme when there is an overabundance of purine triphosphates, and UTP down regulates the enzyme when pyrimidine triphosphates have accumulated to a high level. In addition, UMP kinase is further regulated by divalent metal ions in a novel mechanism related to metal free UTP binding (Serina et al., 1996). The binding of metal-free UTP causes a gel-sol transition that affects the state of UMP kinase aggregation and subsequently the enzyme activity.

In humans, uridine kinase is associated with an autoimmune deficiency that results in susceptibility to respiratory infections such as invasive *Hemophilus influenzae* type B disease in Alaskan Eskimos (Petersen et al., 1985) and South American Indians (Gallango and Suinaga, 1978; Gallango et al., 1978). This under-expression of the UMP kinase results in a syndrome similar to the immune defect resulting from adenosine deaminase deficiency (Giblett et al., 1974), thought to be due to the toxic buildup of substrates.

In plants, UMP kinase has received only marginal study. UMP kinase is elevated during seedling development (Deng and Ives, 1972; Mazus and Buchowicz, 1972) and fruit ontogeny (Deng and Ives, 1975; Rudd and Fites, 1972). Because UMP kinase is likely to be as important in plants as it is in microorganisms, we have isolated the cDNA for the *Arabidopsis thaliana* UMP/CMP kinase, expressed the coding region in *E. coli*, and have characterized the resulting plant enzyme.

Experimental Procedures

Materials

The pGEX-4T-3 and Glutathione Sepharose 4B were purchased from Pharmacia (Pharmacia Biotech, Piscataway, NJ). The vector pT7-Blue was from Novagen (Madison, WI). Enterokinase was from Biozyme Laboratories, (San Diego, CA). Restriction enzymes, T4 Ligase, and Taq polymerase were from Promega (Madison, WI). All other enzymes and reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. The *Arabidopsis thaliana* cDNA library in a yeast transformation vector was previously described (Minet et al., 1992). Oligonucleotides were synthesized at the Iowa State University Nucleic Acid Facility.

Strains

The *S. cerevisiae* strain FL100a was the wild-type strain used for all yeast-related manipulations. The *S. cerevisiae ura6* strain was derived from FL100a and displayed a conditional thermosensitive and 5-fluorouracil-resistant phenotype (Liljelund and Lacroute, 1986). The strain used in these studies was also auxotrophic for histidine and tryptophan. Genotype of the yeast strains were established by plating at either the permissive or restrictive temperature on media lacking various ingredients. The bacterial strain XL1-Blue was used for all bacterial manipulations.

Yeast methods

Yeast transformation was conducted as described (Gietz et al., 1992). *S. cerevisiae* auxotrophic mutants were complemented by *Arabidopsis thaliana* cDNAs was as previously described (Minet et al., 1992). To rescue the plasmids from the yeast, a loop full of yeast cells was suspended in 0.4 ml of 10 mM Tris, pH 8.0, 1 mM EDTA, 0.4 M NaCl, and vortexed (2 min) with 200 µl of glass beads. Following vortexing, an equal volume of phenol/chloroform (1:1) was added and the vortexing was repeated. After centrifugation, 250 µl of supernatant was removed and DNA was precipitated by the addition of 500 µl of ethanol. After washes

with 70% ethanol, the DNA was resuspended in water and used for electroporation of *E. coli* (Ausubel et al., 1993).

Recombinant DNA methods

Sequencing - DNA sequencing reactions were performed using the Applied Biosystems Prism Dye-deoxy Cycle Sequencing Kit. The reactions were run on an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer Corp.). Sequencing was initiated from known vector sequences. On the basis of these runs, oligonucleotide primers specific to the *Arabidopsis thaliana* UMP/CMP kinase sequence were constructed. DNA sequences were performed in duplicate or triplicate for each run. Each strand was completely sequenced including sequencing through all restriction enzyme sites, and the entire sequence of the cDNA was confirmed on the opposite strand.

Vector construction - The 606 bp UMP Kinase coding region was PCR amplified from the pAt-URA6 clone using a pair of oligonucleotides, LZ2080 (5'-GCGGATCCGATGACGATGACAAGATGGGATCTGTTGATGCTGCT-3') and LZ2081 (5'-CGGCTCGAGCTACTAGGCTTCAACCTTCTCAGC-3'). The PCR product was cloned into the pT7-Blue(R) T-vector to form the vector pRT379. These oligonucleotide primers introduced into the PCR product unique *Bam*HI and *Xho*I sites at the ends of the PCR fragment, an enterokinase site for cleavage of the GST fusion protein, and an additional stop codon. Following cloning of the PCR product in pRT379, the sequence of the coding region was confirmed by completely sequencing the insert. The expression vector, pRT380, was subsequently prepared by inserting the 637 bp *Bam*HI/*Xho*I fragment from pRT379 into the *Bam*HI/*Xho*I sites of pGEX-4T-3 to form the vector pRT380. Again the sequence of the coding region was confirmed by completely sequencing the insert.

Expression in *E. coli*

The vector pRT380 was transformed into *E. coli* XL1-Blue for the production of the fusion protein. To induce the fusion protein, 5 ml of overnight culture were diluted into 500

ml of 2YT media and grown at 37°C until mid-log phase, then IPTG was added to a final concentration of 1 mM. Growth continued for 3 h at 37°C. Cells were harvested by centrifugation at 4000 x g for 10 min. The cell pellet was stable when stored at -20°C.

Purification of UMP/CMP kinase

Frozen cells were thawed in 10 ml PBS (phosphate buffered saline) containing 25 mg Lysozyme. After 30 min at room temperature, the cells were sonicated on ice. DNAse I (2 mg) in 0.8 M $MgCl_2$ was added and the cell sonicate was incubated at room temperature for 10 min. Cellular debris was removed by centrifugation (12,000 x g) for 40 min and the supernatant was used to resuspend 0.3 ml of PBS-washed glutathione-sepharose beads. The beads and supernatant were incubated overnight at room temperature on a rotary wheel. The next morning, the beads were removed by centrifugation and washed with PBS until the supernatant was clear (usually 5 times). The fusion protein could be eluted by the addition of 10 mM glutathione; however, for most studies, the UMP/CMP kinase domain was removed from the bound fusion protein. This was done by resuspending the beads in 0.35 ml enterokinase buffer (25 mM Tris pH 7.5 + 10 mM $CaCl_2$) and digesting with 1000 units of enterokinase until completion at room temperature on a rotating wheel. After digestion, the supernatant was recovered by centrifugation and applied to a Vydac reverse phase $C_{(18)}$ HPLC column (250 mm x 10 mm I.D.). The column was eluted at a rate of 4 ml min^{-1} with a programmed elution profile. Solvents used were (A) 0.1% trifluoroacetic acid in water and (B) 0.08% trifluoroacetic acid in acetonitrile. The enzyme was eluted by a series of linear gradients: 0 to 5 min, gradient from 20% B to 40% B; 5 to 23 min, gradient from 40% B to 43% B. The column was cleaned and equilibrated for further separations by two gradients: 23 to 26 min, gradient from 43% B to 100 % B and 26 to 28 min, gradient back to 20% B. The UMP/CMP kinase was eluted at 41% Buffer B. Following elution, the enzyme fraction was dialyzed against 20 mM MES, pH 6.5 at 4°C for 24 h, then concentrated to about 0.5 ml using a Centricon-10 centrifugal concentrator (Amicon, Inc., Beverly, MA).

Protein methods

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. SDS PAGE was performed according to Laemmli (1970). N-terminal amino acid analysis was performed in the Iowa State University Protein Facility by sequential Edman Degradation on an Applied Biosystems 477A protein Sequencer/120A Analyzer. Amino acid composition was performed with PITC derivatized amino acids following hydrolysis of purified UMP/CMP Kinase in 6N HCl.

Matrix-assisted laser desorption ionization (MALDI) Mass Spectroscopy - Protein samples of 0.5 to 1.0 µl, containing about 0.5 to 1 µg of protein, were loaded with 0.5 µl of freshly prepared 3,5-dimethoxy-4-hydroxy cinnamic acid matrix onto a Finnigan LASERMAT 2000 MALDI-time of flight mass analyzer. The collected data were analyzed using the LASERMAT 2000 data processing software. Lysozyme was used as an internal calibration standard.

Molecular weight determination by sedimentation equilibrium - Analytical ultracentrifugation experiments were performed using a Beckman Optima XL-A ultracentrifuge. The temperature of rotor (AN-60 Ti) was maintained at 20°C and speeds were set at 8,000, 10,000 and 14,000 rpm. Samples of protein were prepared in 20 mM MES (pH 6.5) at concentrations of 2.0-5.8 µM corresponding to absorbance of 0.3-0.5 at 230 nm. Protein samples were centrifuged at least 14 h to attain sedimentation equilibrium before data collection. Absorbance readings were measured at 1 h intervals to ensure that equilibrium had been reached. The gradient of protein concentration in the cell was determined by UV absorption at 230 nm by stepwise radial scans and sixty consecutive automated optical scans acquired at each 0.001 cm of radial spacing. The partial specific volume of the UMP/CMP kinase, 0.740 mL/g, was calculated by the method of Cohn and Edsall (1943). Sedimentation equilibrium single data set was analyzed as a single ideal species using the "IDEAL" model on

the Optima XL-A Analysis Software (Version 2.0) to get the molecular weight. Sedimentation equilibrium data obtained at three different rotor speeds were analyzed to obtain the association constant for the monomer-dimer equilibrium using "SELF" model in multiple data set analysis program Optima XL-A.

Kinetic Studies - Enzymatic Activity was determined spectrophotometrically by measuring the formation of ADP and UDP at 23°C with a coupled enzyme assay (Agarwal et al., 1978). Quantitation was performed by following the decrease of NADH absorbance at 340 nm. For kinetic analyses, different concentrations of ATP and UMP were utilized as indicated for each experiment. The initial-rate data were analyzed for kinetic mechanisms (Fromm. 1975) by using a computer program written in the MINITAB language with an α-value of 2.0 (Siano et al., 1975).

Results

Cloning by complementation

Yeast genetic crosses were made between *ura3*$^-$ and *ura6*-15$^{(Ts)}$ to provide progeny blocked in *de novo* pyrimidine biosynthesis as well as blocked in the conversion of UMP into UDP. The inclusion of the *ura3*$^-$ mutation provided a better screen for *ura6*-15$^{(Ts)}$ by reducing the number of false transformants arising from the *ura6*-15$^{(Ts)}$ mutant. These progeny were unable to make pyrimidines via the *de novo* pyrimidine biosynthetic pathway but could be rescued at the permissive temperature by addition of uracil through the salvage pathway. Progeny containing both mutations were selected and the genotypes were established for these progeny. Strain *ura6*-15A was obtained, which had the following genotype: FL100a, *ura6*-15^{Ts}, *ura3*$^-$, *trp1*$^-$, *his3*$^-$. All subsequent transformations were performed with this strain, *ura6*-15A.

The yeast strain was transformed with the *Arabidopsis thaliana* cDNA library in the vector pFL61 as described in the "Experimental Procedures" section. Approximately 54,000 individual transformants were screened for colonies at the restrictive temperature. Two

colonies were found from the 54,000 transformants. After replica plating to insure that the phenotype was indeed correct, DNA was isolated from each of these yeast strains and *E. coli* were transformed by electroporation. Both colonies yielded the identical cDNA clone when analyzed by DNA sequence analysis and were taken to be duplicate colonies. Thus, the cloning frequency of the UMP/CMP Kinase cDNA was 1/27,000. After growth in *E. coli*, both clones were reisolated and used to transform the original *ura*6-15A strain to verify that the clones produced the Ura$^+$ phenotype in the mutant. The clone was termed pAt-ura6.

Analysis of the UMP/CMP kinase mRNA

The DNA sequence of the pAt-ura6 insert was completely established and is presented in Figure 1. The *Arabidopsis thaliana* UMP/CMP Kinase cDNA sequence has been deposited in the GenBank as accession number AF000147. This cDNA is 895 nucleotides long. It shows a 60 nucleotide 5' untranslated region, a coding region of 606 nucleotides encoding a 202 amino acid protein and a 175 nucleotide 3' untranslated region extending to the poly A site at position 874. Interestingly, this cDNA does not show a typical polyadenylation signal. The closest to the consensus is AATTTT which is duplicated at positions 836..841 and 853..858. DNA matrix analysis revealed no significant regions of internal duplication or inverted repeats within the cDNA sequence.

The translation start codon correlates well with Kozak's rules (Kozak, 1986). The sequence at the translation start differs from the eukaryotic consensus at only a single nucleotide (ACAATGG); consequently, this cDNA is expected to be translated very efficiently.

Analysis of the UMP/CMP kinase protein

The translated amino acid sequence of the *Arabidopsis* UMP/CMP kinase is also presented in Figure 1. The protein is a 202 amino acid polypeptide. There is a pair of conserved cysteine residues which are shared among all of the eukaryotic UMP kinases;

however, it has been proposed that the conserved cysteine residue found at position 31 in the *Arabidopsis* protein has a free SH group (Weismüller et al., 1990).

When the amino acid sequence of the *Arabidopsis* UMP/CMP kinase was compared with the sequences of other pyrimidine kinases we observed that the plant enzyme is very similar to UMP kinases from other eukaryotic sources (Figure 2). UMP kinases have been isolated from yeast (*S. cerevisiae*), a cellular slime mold (*D. discoideum*), and a mammal (*S. scrofa*). Each of these enzymes shows 47 to 53% amino acid identity with each other. Interestingly, 32.2% of the amino acid residues are identical among all of the four proteins indicating that the eukaryotic protein is highly conserved from all sources. If conservative substitutions are permitted then the identity among the four proteins rises to 47.5% identity. In contrast, the UMP kinases from bacterial or archebacterial sources are more similar to the eukaryotic TMP kinases than they are to the eukaryotic UMP kinases.

Among the conserved residues is a region near the N-terminus that is maintained in nucleotide-binding proteins (Möller and Amons, 1985). This sequence, GGPG$^{S}_{A}$GK, is also preserved in the eukaryotic nucleotide monophosphokinases. In crystallographic studies of adenylate kinase Pai et al., (1977) found that this loop anchors the γ-phosphate moiety of ATP.

Expression in *E. coli*

After characterization of the *Arabidopsis* UMP/CMP kinase cDNA, we constructed a vector to express the *Arabidopsis* protein in *E. coli*. Details of this construction are outlined in "Experimental Procedures". Figure 3, Panel A shows the structure of the GST fusion protein used in this work. Also, the nucleotide sequence of the GST-kinase and the kinase-vector junction in pRT380 are presented in Panel B. Because the plant UMP/CMP kinase does not contain an N-terminal signal sequence, the vector was designed so that enterokinase would cleave at the exact N-terminus of the enzyme to yield the full-length UMP/CMP kinase protein.

After transfer of the vector to XL1-Blue cells, the induction of these cells by IPTG resulted in accumulation of a 50 KDa protein that was not present in host cells without plasmid

(data not shown) or uninduced cells containing pRT380 (Lane 1, Figure 4). After sonication of the IPTG-induced cells, enzymatic assays showed the presence of a new UMP kinase activity that was not present in control cells (data not shown). This new UMP kinase activity arises from the GST-kinase fusion protein.

Purification of UMP/CMP kinase

The various steps in the purification of the enzyme were monitored by SDS PAGE as shown in Figure 4. A 51 KDa protein was observed upon induction of the cells containing pRT380 with IPTG (compare Lane 2, induced, to Lane 1, uninduced). This fusion protein could be selectively bound and eluted from the glutathione-Sepharose beads (Lane 3). Enterokinase digestion of the fusion protein resulted in the conversion of the 51 KDa protein into two proteins of 29 KDa and 22 KDa (Lane 4). For most studies, however, the GST-kinase fusion protein was proteolyzed while still bound to glutathione-sepharose beads. This releases the 22 KDa protein while the 29 KDa protein remains bound to the Sepharose (Lane 5). After enzymatic cleavage of the fusion protein the UMP/CMP kinase was purified by HPLC. A typical HPLC elution profile is shown in Figure 5. The eluted UMP/CMP kinase protein was collected, dialyzed, and concentrated. An aliquot of the purified UMP/CMP kinase after HPLC elution is shown in Lane 6 of Figure 4.

About 0.5 to 1 μg of the HPLC purified UMP/CMP kinase was subjected to MALDI mass spectrometry (Figure 6). The subunit molecular mass of the purified UMP/CMP kinase $[MH]^+$ peak by MALDI analysis was 22,405 ± 185 daltons (n = 4). Secondary peaks $[MH_2]^{++}$ and $[2MH]^+$ were identified at 11,286 ± 94 daltons and 44,894 ± 333 daltons. Therefore, the subunit molecular mass found from the average of these masses (22,448 ± 44), is in excellent agreement with the molecular mass of UMP/CMP kinase predicted from the cDNA (22,482 Da).

Characterization of the purified UMP/CMP kinase protein

To confirm that the expressed and purified protein was indeed the UMP/CMP kinase we chose to perform two analyses. First, the N-terminus of the purified protein was sequenced. An aliquot of the purified protein was run on a 14% SDS PAGE gel and then the protein was electroblotted to PVDF membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was stained with Coomassie-Blue R250. The single band was cut from the PVDF membrane and the N-terminal sequence was determined by Edman degradation at the Iowa State University Protein Facility using an Applied Biosystems model 477A protein sequencer and a 120A PTH amino acid analyzer. The N-terminal sequence of the 22 KDa protein was MGSVDAANGSGK... This is the identical amino acid sequence of the sequence predicted from the cDNA (Compare with Figure 1). This sequence indicates not only that the N-terminus of the purified 22 KDa protein is identical with the UMP/CMP kinase protein, but also that the enterokinase cuts exactly at the predicted site leaving no extra amino acids present on the N-terminus of the expressed kinase protein.

The amino acid composition of the expressed protein was also determined. About 1 µg of HPLC purified UMP/CMP kinase was hydrolyzed in 6N HCl for 65 min at 150°C in a Waters PICO-TAG™ workstation. The free amino acids were derivatized under basic conditions with phenyl isothiocyanate in an Applied Biosystems model 420A derivatizer and separated on a narrow-bore C_{18} column. The phenylthiocarbamyl chromophore was detected at 254 nm. Quantitation was performed from the absorbance at 254 nm and comparison to a norleucine internal standard. The total amino acid composition was twice determined independently. These analyses are presented in Table 1. Gln and Asn could not be detected because both amino acids were converted to the corresponding carboxylic acids. Thus, the resulting Asp and Glu were the sum of Asp and Asn (Asx) and of Glu and Gln (Glx), respectively. The number of Trp and Cys residues could not be determined by this method

because they were destroyed during acid hydrolysis. As seen in Table 1, there was good agreement between the independent analyses. The composition of the UMP/CMP kinase predicted from the cDNA sequence is also presented in Table 1. A χ^2 analysis indicated with greater than 99% confidence that the composition found in the purified protein is the same as that of the predicted protein confirming that the purified protein is the *Arabidopsis thaliana* UMP/CMP kinase.

Initial rate studies

Once we were convinced that the purified protein was indeed the *Arabidopsis thaliana* UMP/CMP kinase, the kinetic parameters of this purified protein were evaluated. After optimization for ATP and UMP concentration, the kinetic parameters of the purified enzyme were determined (Table 2). As can be seen from this Table, the *Arabidopsis thaliana* UMP/CMP kinase can utilize CMP as a phosphate donor almost as well as it does UMP. The K_m for UMP was 5 to 6 fold higher when compared with that of ATP. Thus, the enzyme binds ATP much more tightly than UMP; however, the K_m for CMP was about equal to the K_m for ATP indicating that the enzyme binds CMP as tightly as ATP. The k_{cat} obtained for each substrate is relatively the same.

pH dependence of the enzyme

The values of k_{cat} were determined at a series of different pH values from 5.5 to 8.0. All pH buffers contained both 50 mM MES and 50 mM Hepes. When ATP was the variable substrate, the concentration of UMP was fixed at 400 μM. When UMP was the variable substrate, the concentration of ATP was fixed at 300 μM. The values of k_{cat} were obtained at pH 5.5, 6.5, 7.0, and 7.5 and 8.0, when ATP or UMP was the variable substrate (data not shown). These analyses showed that optimal activity was achieved at pH 6.5 for both ATP and UMP.

Thermal stability

The enzyme was heated for 10 min at various temperatures between 30°C and 100°C. Then residual activity was determined with 300 μM ATP and 400 μM UMP (data not shown). Half of the enzymatic activity was maintained when the enzyme was heated at 58°C. Although still high, this is about 10°C lower than that of the *E. coli* enzyme.

Subunit structure

Equilibrium sedimentation was conducted at three different concentrations and three different centrifugation speeds. The data showed that the molecular weight of UMP/CMP kinase does not depend on either the protein concentrations or the centrifugation speeds under the conditions tested. Typical sedimentation equilibrium data are shown in Figure 7. In Figure 7c, at low concentrations, the apparent molecular weight was 22 KDa, which is very close to the known mononer value determined by MALDI or the cDNA sequence. The highest concentration had an apparent molecular weight of approximately 45 KDa, which indicates a monomer-dimer equilibrium system. The native molecular mass of UMP/CMP kinase was found to be 44,697 ± 1,912 (n = 5). Thus, the likely structure of the *Arabidopsis* UMP/CMP kinase is a dimer, composed of two identical subunits. The association constant for the monomer-dimer equilibrium was 76.6 (in 230 nm absorbance units, goodness of fit = 0.65, determined by the χ^2 test).

Kinetic mechanism

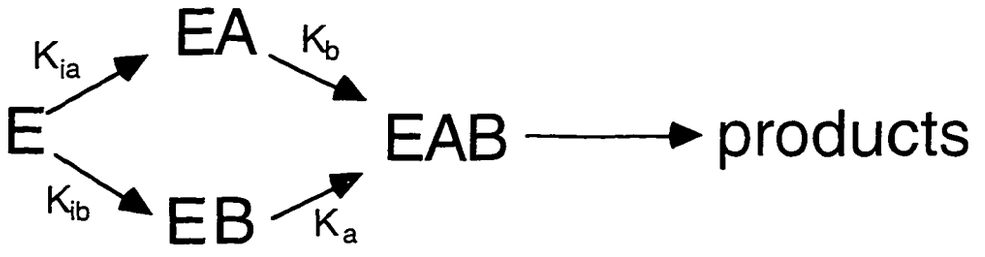
To better understand the kinetic mechanism, a series of enzyme assays were conducted in which one substrate was varied at different fixed concentrations of other substrates. Double reciprocal plots of these data showed that when the ATP concentration was varied at different fixed levels of UMP, a family of lines intersecting in the second quadrant was obtained (Figure 8, Panel A). A similar family of lines was obtained for various 1/UMP concentrations at different fixed levels of ATP (Panel B). These families of lines shown in both panels of Figure

8 are theoretical; fit to Equation 1 when n = 1. The data obtained from the experimental evaluation matches the theoretical family of lines for both ATP and UMP. Based on the closeness of this fit, we conclude that the enzyme fits a random Bi-Bi mechanism. The data fit the random Bi-Bi mechanism shown in Eq. 1 (Fromm, 1975):

$$\frac{1}{V} = \frac{1}{V_m}\left[1+\frac{K_a}{A^n}+\frac{K_b}{B}+\frac{K_{ia}K_b}{A^nB}\right] \qquad \text{(Eq. 1)}$$

where V, V_m, A, B, K_a, K_b, and K_{ia} represent the initial velocity, maximum velocity, concentration of free ATP, concentration of free UMP, Michaelis constant for ATP, Michaelis constant for UMP, and dissociation constant for ATP, respectively. n represents the Hill coefficient for ATP. K_{ib} is the dissociation constant for UMP. Equation 1 is the fundamental initial rate equation for the sequential kinetic mechanism shown in Scheme 1. K_{ia} and K_{ib} were determined experimentally. K_{ia} (for ATP) = 14.9 ± 1.3 μM and K_{ib} (for UMP) = 110.2 ± 8.7 μM.

Scheme 1:



$$E + A = EA,\ K_{ia} = \frac{[E][A]}{[EA]} \qquad \text{(Eq. 2)}$$

$$E + B = EB,\ K_{ib} = \frac{[E][B]}{[EB]} \qquad \text{(Eq. 3)}$$

$$EA + B = EAB,\ K_b = \frac{[EA][B]}{[EAB]} \qquad \text{(Eq. 4)}$$

$$EB + A = EAB,\ K_a = \frac{[EB][A]}{[EAB]} \qquad \text{(Eq. 5)}$$

$K_{ia}K_b = K_aK_{ib}$

Alternative substrates

To examine the specificity of the UMP/CMP kinase in more detail, various phosphate donors and acceptors were evaluated for their ability to function in this enzyme assay. To evaluate the phosphate acceptors, 300 μM ATP was used as the donor with different monophosphate acceptors at 400 μM. As shown in Table 3, only UMP and CMP are effective phosphate acceptors. Neither OMP nor TMP were effective as a phosphate acceptor. The presence of the 2' hydroxyl on the ribose moiety is also important because dUMP and dCMP are 30-fold less active than their ribosyl analogs. None of the purine monophosphates tested functioned effectively as phosphate acceptors.

Similarly, other nucleotide triphosphates were examined for their ability to function as a phosphate donor to UMP. To evaluate the phosphate donors, 300 μM donors were used with UMP at 400 μM. In this analysis, only ATP and dATP were effective phosphate donors with dATP only half as effective as ATP. Other purine triphosphates were 20- to 30-fold less active than ATP. Pyrimidine triphosphates were 70-fold less active than ATP.

It is known that the *E. coli* UMP kinase is allosterically regulated by both GTP and UTP (Serina et al., 1995). With the *E. coli* enzyme, 100 μM GTP activates the enzyme five-fold and 100 μM UTP down-regulates the enzyme five-fold. We therefore explored whether these nucleotides affected the enzyme activity of the *Arabidopsis* enzyme. Neither of these nucleotides significantly affects the activity of the *Arabidopsis* UMP/CMP kinase even at extremely high levels (Table 4). GTP does indeed increase the enzyme activity, but only by 25% as compared with the 5-fold activation of the *E. coli* enzyme with much lower levels of GTP (Serina et al., 1995). Conversely, UTP also affects the UMP/CMP kinase activity, but again only by 25%. Thus, whereas GTP and UTP both affect the plant enzyme, the effect of each of these nucleotides even at very high levels is significantly less than the effect on the prokaryotic enzyme.

Kinetics of AP_5A inhibition

Finally we examined the inhibition of the *Arabidopsis thaliana* UMP/CMP kinase with the bifunctional inhibitor AP_5A (Figure 9). The I_{50} value was determined by fixing ATP at 300 μM and UMP at 400 μM and varying the AP5A concentration between 0 and 100 μM. The I_{50} value of AP5A on the *Arabidopsis* enzyme was 14 μM, which is lower than that found on the enzyme from either *D. discoideum* (Weismüller et al., 1990) or from *E. coli* (Serina et al., 1995). The inhibition mechanism of AP5A was determined by fixing one substrate at a saturating concentration and varying the concentration of the other substrate at different fixed concentrations of AP5A. The families of lines shown in Figure 9 are theoretically fit to Equation 6 (Fromm, 1975) and the points are experimentally derived.

$$\frac{1}{V} = \frac{1}{V_m}\left[1+\frac{K_a}{A}\left(1+\frac{I}{K_i}\right)\right] \quad \text{(Eq. 6)}$$

where V, V_m, A, I, K_a, and K_i represent the initial velocity, maximum velocity, concentration of free ATP (or UMP), concentration of free AP5A, Michaelis Menton constant for ATP (or UMP), and inhibition constant for AP5A, respectively.

Panel A shows the inhibition by AP5A when ATP was the variable substrate and Panel B shows the inhibition of AP5A when UMP was the variable substrate. These data clearly demonstrate that AP5A is a competitive inhibitor of both ATP and UMP. The K_is for ATP and UMP were 1.20 ± 0.02 μM and 6.53 ± 0.03 μM, respectively.

Discussion

We have isolated the cDNA encoding the *Arabidopsis thaliana* UMP/CMP kinase by complementation of a *S. cerevisiae* UMP kinase mutant. Complementation of known yeast mutants is a very effective way to obtain eukaryotic genes. Following isolation, the plant cDNA was characterized by sequencing. The full-length cDNA encoded a 202 amino acid protein that is closely related to UMP kinases from other eukaryotic sources. The plant

enzyme, however, did not share high identity with the bacterial or archebacterial UMP kinases. The prokaryotic UMP kinases present in the GenBank were found to be more closely related to eukaryotic TMP kinases than they were to the eukaryotic UMP kinase. In previous studies, the *E. coli* UMP kinase was found to belong to the aspartokinase family (Serina et al., 1995).

When the intracellular location of the *Arabidopsis* uridine kinase was examined using the online analysis tool PSORT at [http://psort.nibb.ac.jp/] (Nakai and Kanehisa, 1992), this tool predicted that this enzyme is cytosolic. A cytosolic localization has also been found for the rice adenylate kinase enzyme (Kawai and Uchimiya, 1995). The cytosolic localization is also consistent with the finding that uridine nucleotides are predominantly located in the cytosol (Dancer et al., 1990).

After characterization of the cDNA, the coding region was expressed by fusing it to glutathione-S-transferase. Following expression, the fusion protein was cleaved and the *Arabidopsis* UMP/CMP kinase was purified by HPLC. The molecular mass, the amino acid composition and the N-terminal sequence of the expressed protein all demonstrated that the expressed protein was indeed UMP/CMP kinase. When examined for enzyme activity, the purified enzyme was found to have both UMP kinase and CMP kinase activity. Kinetic parameters were determined for the plant enzyme and several differences were noted with the *E. coli* enzyme.

First, the *E. coli* enzyme shows remarkable thermal stability in the absence of protective agents. The *E. coli* enzyme is stable up to 65°C. Although relatively thermostable, the *Arabidopsis* enzyme did not show the same degree of stability as the *E. coli* enzyme. Second, the *E. coli* enzyme is a hexamer of 26 KDa subunits, whereas the *Arabidopsis* enzyme is a dimer. Finally, the plant enzyme is not allosterically regulated in the same way that the prokaryotic enzyme is regulated. At concentrations that dramatically affect the enzyme activity of the *E. coli* UMP kinase, neither GTP nor UTP have a significant effect on the plant enzyme. Indeed at 30 times these levels, the plant enzyme is only affected by about 25%. Bourne *et al.*,

(1991) have identified a pair of sequences ^{77}Asp-His-Met-Gly^{80} and ^{165}Thr-Lys-Val-Asp^{168} that are conserved in GTP binding proteins. Both of these sequences are conserved in the *E. coli* UMP kinase (Serina et al., 1995); however, neither of them are present in the *Arabidopsis* UMP/CMP kinase. Thus, by both sequence identity and experimental observation, allosteric regulatory sites are not present in the plant enzyme, and the *Arabidopsis* enzyme seems to be both functionally and structurally different from the prokaryotic enzyme.

The K_m values for UMP and CMP indicate that the *Arabidopsis* enzyme can utilize both pyrimidine monophosphates equally well as phosphate acceptors. This is similar to the enzyme from Rat Novikoff ascites tumors (Orengo and Maness, 1978) whereas the mouse enzyme utilizes uridine nearly twice as effectively as cytidine as a phosphate acceptor (Andersen, 1978a). The deoxy forms, however, are almost totally ineffective as phosphate acceptors. Two important conclusions can be drawn from this finding: first, a different enzyme must be responsible for the conversion of dCMP into the dCDP. This is different from the mouse and the *Tetrahymena* enzyme, where dCMP can also act as a phosphate acceptor (Andersen 1978b). Secondly, because the nucleotide monophosphate binding pocket does not discriminate between UMP and CMP, the exclusion of deoxynucleotides results not only in the exclusion of dCMP but also in the exclusion of dUMP. Thus, the conversion of dUMP into dUDP does not occur, thereby forcing the conversion of dUMP into TMP by thymidylate synthase. Further, TMP is ineffective as a phosphate acceptor, indicating that a separate TMP kinase must also exist as in yeast (Jong et al., 1984). Structural studies of adenylate kinase from both *E. coli* (Müller and Schulz, 1992) and beef heart (Diederichs and Schulz, 1991) revealed that the 2' hydroxyl of the phosphate acceptor forms a strong hydrogen bond with an α-chain carboxyl. It is likely that similar interactions are required in the plant UMP/CMP kinase thereby biasing the specificity against the deoxynucleotides.

Eukaryotic uridine kinases, in general, have a higher specificity for ATP as the phosphate donors with dATP effective at about 10% the level of ATP (Orengo and Maness,

1978). This was also found for the plant enzyme. Other nucleotide triphosphates were essentially ineffective as phosphate donors.

The plant uridine kinase has a high degree of identity with other eukaryotic uridine kinases. Therefore, it is expected that the plant enzyme also shares significant structural identity with other eukaryotic uridine kinases. The uridine kinase enzyme from yeast has been purified and characterized (Ma et al., 1990). The crystal structure of this enzyme has been solved with substrates in place (Müller-Dieckmann and Schulz, 1994, 1995). The substrates are held in position by numerous favorable contacts with the protein. Most of these contacting residues are conserved between the yeast enzyme and the plant enzyme.

These studies have also demonstrated that the UMP binding pocket of the yeast enzyme is of sufficient size to accommodate an AMP moiety. This explains the high activity of the yeast UMP kinase for AMP. The finding that AP_5A is a competitive inhibitor of UMP, with a micromolar K_i, indicates that the *Arabidopsis* enzyme has a UMP binding pocket that is also sufficiently large to accommodate an adenine moiety. The *Arabidopsis* enzyme; however, has < 0.5% activity with AMP. Therefore, the structure of the UMP binding pocket of the *Arabidopsis* enzyme will be particularly interesting to understand. The X-ray crystallographic studies of the yeast UMP kinase fail to explain the specificity of this enzyme for UMP. Those residues that have been shown to line the uracil binding pocket of the yeast UMP kinase [Ala^{47}, Leu^{51}, Ile^{75}, Val^{76}, Thr^{81}, Phe^{105}, Arg^{107}, and Gln^{111}] are, with only one exception [Asn substitutes for Gln], completely conserved in the *Arabidopsis* enzyme. Note that this same substitution [Asn for Gln] is also found in the *Dictyostelium discoideum* enzyme, which also shows a high degree of substrate discrimination for UMP over AMP (Weismüller et al., 1990).

The enzymatic mechanism is also relatively well understood for the yeast enzyme. The transition state of phosphoryl transfer is maintained by a scaffold of interactions. These interactions include the C^{α}-backbones of the CORE domains and a series of six positively charged residues [Lys^{29}, Arg^{52}, Arg^{107}, Arg^{142}, Arg^{148}, Arg^{159}] positioned to coordinate the

phosphates. All of these residues are conserved in the *Arabidopsis* enzyme. The interaction of the substrate-fixed phosphates with the yeast UMP kinase is virtually identical with the *E. coli* adenylate kinase (Müller-Dieckmann and Schultz, 1994) indicating the widespread and general conservation of this enzymatic mechanism. Because of the high degree of conservation in these important contacting residues, it is probable that the *Arabidopsis* enzyme also shares this enzymatic mechanism.

Acknowledgment

The authors would like to thank Dr. Michelle Minet for her help in yeast molecular biology techniques and Dr. Herbert Fromm for his help in analysis of the kinetics of UMP/CMP kinase.

References

Agarwal, K.C., Miech, R.P. and Parks, R.E. (1978) Guanylate kinase from human erythrocytes, hog brain and rat liver. Methods Enzymol. 51, 483-490.

Anderson, E.P. (1978b) UMP-CMP kinase from *Tetrahymena pyriformis*. Methods Enzymol. 51, 331-337.

Anderson, E.P. (1978a) Uridine-cytidine kinase from a murine neoplasm. Methods Enzymol. 51, 314-321.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1993) Current Protocols in Molecular Biology. John Wiley & Sons, New York, pp.1.8.4-1.8.5.

Baranowska, H., Zaborowska, D., Jachymczyk, W.J. and Zuk, J. (1990) Role of the *CDC8* gene in the repair of single strand breaks in DNA of the yeast *Saccharomyces cerevisiae*. Curr. Genet. 18, 175-179.

Baranowska, H. and Zuk, J. (1991) Chemical mutagenesis and DNA synthesis in *cdc8*, a DNA replication mutant of *Saccharomyces cerevisiae*. Curr. Genet. 20, 471-474.

Birkenmeyer, L.G., Hill, J.C. and Dumas, L.B. (1984) *Saccharomyces cerevisiae CDC8* gene and its product. Mol. Cell. Biol. 4, 583-590.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. Nature 349, 117-127.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72, 248-254.

Cohn, E.J. and Edsall, J.T. (1943) Proteins, amino acids and peptides as ions and dipolar ions. Rheinhold, New York, pp. 157.

Dancer, J., Neuhaus, H.E. and Stitt, M. (1990) Subcellular compartmentation of uridine nucleotides and nucleoside-5'-diphosphate kinase in leaves. Plant Physiol. 92, 637-641.

Deng, Q.-I. and Ives, D.H. (1972) Modes of nucleoside phosphorylation in plants: Studies on the apparent thymidine kinase and true uridine kinase of seedlings. Biochem. Biophys. Acta 277, 235-244.

Deng, Q.-I. and Ives, D.H. (1975) Non-allosteric regulation of the uridine kinase from seeds of *Zea mays*. Biochem. Biophys. Acta 377, 84-94.

Diederichs, K. and Schulz, G.E. (1991) The refined structure of the complex between adenylate kinase from beef heart mitochondrial matrix and its substrate AMP at 1.85 Å resolution. J. Mol. Biol. 217, 541-549.

Fromm, H.J. (1975) Initial Rate Enzyme Kinetics. Springer-Verlag, Berlin, pp. 36-37.

Gallango, M.L. and Suinaga, R. (1978) Uridine monophosphate kinase polymorphism in two Venezuelan populations. Am. J. Hum. Genet. 30, 215-218.

Gallango, M.L., Muller, A. and Suinaga, R. (1978) Biochemical characterization of a red cell UMP kinase variant found in the Warao Indians of Venezuela. Biochem. Genet. 16, 1085-1093.

Giblett, E.R., Anderson, J.E., Chen, S.-H., Teng, Y.-S., and Cohen, F. (1974) Uridine monophosphate kinase: a new genetic polymorphism with possible clinical implications. Am. J. Hum. Genet 26, 627-635.

Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. Nucl. Acids Res. 20, 1425-1428.

Jong, A.Y., Kuo, C.-L. and Campbell, J.L. (1984) The CDC8 gene of yeast encodes thymidylate kinase. J. Biol. Chem. 259, 11052-11059.

Jong, A., Yeh, Y. and Ma, J.J. (1993) Characteristics, substrate analysis and intracellular location of *Saccharomyces cerevisiae* UMP kinase. Arch. Biochem. Biophys. 304, 197-204.

Kawai, M. and Uchimiya, H. (1995) Biochemical properties of rice adenylate kinase and subcellular location in plant cells. Plant Mol. Biol. 27, 943-951.

Kozak, M. (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44, 283-292.

Kuo, C.L. and Campbell, J.L. (1983) Cloning of *Saccharomyces cerevisiae* DNA replication genes: Isolation of the *CDC8* gene and two genes that compensate for the *cdc8-1* mutation. Mol. Cell. Biol. 3, 1730-1737.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Liljelund, P. and Lacroute, F. (1986) Genetic characterization and isolation of the *Saccharomyces cerevisiae* gene coding for uridine monophosphokinase. Mol. Gen. Genet. 205, 74-81.

Ma, J.J., Huang, S.H. and Jong, A.Y. (1990) Purification and characterization of *Saccharomyces cerevisiae* uridine monophosphate kinase. J. Biol. Chem. 265, 19122-19127.

Mazus, B. and Buchowicz, J. (1972) Activity of enzymes involved in pyrimidine metabolism in the germinating wheat grains. Phytochem 11, 77-82.

Minet, M., Dufour, M.-E. and Lacroute, F. (1992) Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J. 2, 417-422.

Möller, W. and Amons, R. (1985) Phosphate-binding sequences in nucleotide binding proteins. FEBS Lett 186, 1-7.

Müller, C.W. and Schulz, G.E. (1992) Structure of the complex between adenylate kinase from *Escherichia coli* and the inhibitor AP5A refined at 1.9 Å resolution. J. Mol. Biol. 224, 159-177.

Müller-Dieckmann, H.J. and Schultz, G.E. (1994) The structure of uridylate kinase with its substrates, showing the transition state geometry. J. Mol. Biol. 236, 361-367.

Müller -Dieckmann, H.J. and Schultz, G.E. (1995) Substrate specificity and assembly of the catalytic center derived from two structures of ligated uridylate kinase. J. Mol. Biol. 246, 522-530.

Nakai, K. and Kanehisa, M. (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics 14, 897-911.

Newlon, C.S. and Fangman, W.L. (1975) Mitochondrial DNA synthesis in cell cycle mutants of *Saccharomyces cerevisiae*. Cell 5, 423-428.

Orengo, A. and Maness, P. (1978) Pyrimidine nucleoside monophosphate kinase from rat liver and rat Novikoff ascites hepatoma. Methods Enzymol. 51, 321-331.

Pai, E.F., Sachsenheimer, W., Schirmer, R.H. and Schulz, G.E. (1977) Substrate positions and induced-fit in crystalline adenylate kinase. J. Mol. Biol. 114, 37-45.

Prakash, L., Hinkle, D. and Prakash, S. (1979) Decreased UV mutagenesis in *cdc8*, a DNA replication mutant of *Saccharomyces cerevisiae*. Mol. Gen. Genet. 172, 249-258.

Petersen, G.M., Silimperi, D.R., Scott, E.M., Hall, D.B., Rotter, J.I. and Ward, J.I. (1985) Uridine monophosphate kinase 3: a genetic marker for susceptibility to *Haemophilus influenzae* type B disease. Lancet II, 417-418.

Rudd, T.P. and Fites, R.C. (1972) Association of thymidine and uridine kinase activities with changes in nucleic acid levels during peanut fruit ontogeny. Phytochem. 11, 1631-1636.

Schricker, R., Magdolen, V., Kaniak, A., Wolf, K. and Bandlow, W. (1992) The adenylate kinase family in yeast: identification of URA6 as a multicopy suppressor of deficiency in major AMP kinase. Gene 122, 111-118.

Seagrave, J. and Reyes, P. (1987) Pyrimidine nucleoside monophosphate kinase from rat bone marrow cells: A kinetic analysis of the reaction mechanism. Arch. Biochem. Biophys. 254, 518-525.

Serina, L., Blondin, C., Krin, E., Sismeiro, O., Danchin, A., Sakamoto, H., Giles, A.-M. and Bârzu, O. (1995) *Escherichia coli* UMP-kinase, a member of the aspartokinase family is a hexamer regulated by Guanine nucleotides and UTP. Biochem. 34, 5066-5074.

Serina, L., Bucurenci, N., Giles, A.-M., Surewicz, W.K., Favian, H., Mantsch, H.H., Takahashi, M., Petrescu, I., Batelier, G. and Bârzu, O. (1996) Structural properties of UMP-kinase from *Escherichia coli:* Modulation of protein solubility by pH and UTP. Biochem. 35, 7003-7011.

Siano, D.B., Zyskind, J.W. and Fromm, H.J. (1975) A computer program for fitting and statistically analyzing initial rate data applied to Bovine hexokinase type III isozyme. Arch. Biochem. Biophys. 170, 587-600.

Valentin-Hansen, P. (1978) Uridine-cytidine kinase from *Escherichia coli.* Methods Enzymol. 51, 308-314.

Weismüller, L., Noegel, A.A., Bârzu, O., Gerisch, G. and Schleicher, M. (1990) cDNA derived sequence of UMP-CMP kinase from *Dictyostelium discoideum* and expression of the enzyme in *Escherichia coli.* J. Biol. Chem. 265, 6339-6345.

Yamanaka, K., Ogura, T., Niki, H. and Hiraga, S. (1992) Identification and characterization of the *smbA* gene, a suppressor of the *mukB* null mutant of *Escherichia coli.* J. Bacteriol. 174, 7517-7526.

Table 1

Expected and Determined Amino acid composition of the purified UMP/CMP kinase

Amino Acid	Predicted	Found	Amino Acid	Predicted	Found
Alanine	15	15.8 15.8	Lysine	20	23.4 22.8
Arginine	10	10.2 9.9	Methionine	4	3.3 3.1
Aspartic Acid	17	16.5 17.4	Phenylalanine	11	9.9 10.1
Cysteine	2	ND[a]	Proline	9	8.9 9.1
Glutamic Acid	29	32.7 32.7	Serine	9	10.7 10.6
Glycine	18	19.9 20.2	Threonine	7	6.7 6.3
Histidine	3	2.8 2.7	Tryptophan	0	ND[a]
Isoleucine	16	12.6 12.6	Valine	14	11.3 11.6
Leucine	13	10.8 10.8	Tyrosine	5	5.0 5.1

[a]ND = Not detected.

χ^2 = 3.40; with 15 degrees of freedom indicates a >99% confidence level

Table 2

Kinetic parameters of Arabidopsis thaliana UMP/CMP kinase

The standard enzyme reaction contained 50 mM MES, pH 6.5, 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenoylpyruvate, 0.2 mM NADH, 3.5 units of pyruvate kinase, and 5 units of lactate dehydrogenase in a final volume of 1 ml. When ATP was used as the variable substrate, UMP and CMP were fixed at 400 µM and 450 µM, respectively. When UMP or CMP were used as the variable substrate, ATP was fixed at 300 µM for UMP and at 800 µM for CMP. The reaction was started by addition of UMP/CMP kinase. The change in optical density at 340 nm was recorded. One unit of UMP kinase is defined as the amount of enzyme that catalyzed the formation of 1 µmol of UDP or CDP per min.

	Specific Activity (units/mg X 10^{-2})	ATP K_m (µM)	UMP K_m (µM)	CMP K_m (µM)	k_{cat} (s^{-1})
UMP kinase	3.67 ± 0.12	29.3 ± 2.8	152.9 ± 14.5	- - -	7.64 ± 0.24
CMP kinase	4.12 ± 0.05	291.7 ± 24.3	- - -	266.4 ± 21.7	8.57 ± 0.11

Table 3

Substrate Specificity

The standard enzyme reaction contained 50 mM MES, pH 6.5, 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenoylpyruvate, 0.2 mM NADH, nucleotides, 3.5 units of pyruvate kinase and 5 units of lactate dehydrogenase in a final volume of 1 ml. . When evaluating the phosphate acceptor we used 300 μM ATP-Mg^{2+} and 400 μM NMP. When evaluating the phosphate donor, we used 300 μM NTP-Mg^{2+} and 400 μM UMP. The reaction was started by addition of UMP/CMP kinase. One unit of UMP kinase is defined as the amount of enzyme that catalyzed the formation of 1 μmol of UDP per min.

Phosphate Donor	Phosphate Acceptor	Relative Activity	Phosphate Donor	Phosphate Acceptor	Relative Activity
ATP	UMP	100 %	ATP	UMP	100 %
ATP	CMP	96.5	GTP	UMP	5.0
ATP	OMP	0.7	ITP	UMP	3.5
ATP	TMP	0.2	XTP	UMP	2.7
ATP	dUMP	2.7	dATP	UMP	43.2
ATP	dCMP	3.3	dGTP	UMP	1.4
ATP	AMP	0.3	UTP	UMP	1.6
ATP	GMP	0.2	TTP	UMP	1.2
ATP	IMP	0.2	CTP	UMP	1.5
ATP	XMP	0.5	dCTP	UMP	1.3

Table 4

Effect of GTP and UTP on UMP kinase activity

The standard enzyme reaction contained 50 mM MES, pH 6.5, 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenoylpyruvate, 0.2 mM NADH, and 300 μM ATP-Mg^{2+}, 400 μM UMP, 3.5 units of pyruvate kinase and 5 units of lactate dehydrogenase in a final volume of 1 ml. The reaction was started by addition of UMP/CMP kinase.

GTP μM	Vo (/sec X 10^{-3})	Relative Activity	UTP μM	Vo (/sec X 10^{-3})	Relative Activity
0	14.35 ± 0.33	100.0	0	13.37 ± 0.05	100.0
200	15.46 ± 0.02	107.8	200	13.22 ± 0.11	98.9
400	16.41 ± 0.17	114.4	400	13.04 ± 0.37	97.5
1000	17.68 ± 0.48	123.2	1000	12.19 ± 0.14	92.7
1500	15.96 ± 0.10	111.2	1500	11.85 ± 0.04	88.6
2000	16.59 ± 0.23	114.6	2000	11.65 ± 0.25	87.1
2500	15.57 ± 0.03	115.4	2500	11.22 ± 0.01	83.9
3000	15.54 ± 0.22	108.4	3000	10.93 ± 0.04	81.7

Figure Legends

Figure 1. DNA sequence of the *Arabidopsis thaliana* UMP/CMP kinase.

The nucleotide sequence of the *Arabidopsis thaliana* UMP/CMP kinase is presented. Nucleotide number is presented at the end of each row. The amino acids translated from the nucleotide sequence are presented below the nucleotide sequence and the amino acid number is presented above the nucleotide sequence.

Figure 2. Comparison of pyrimidine monophosphokinases.

Panel A. A comparison of the amino acid identity between the various pyrimidine monophosphate kinases was performed using the Wisconsin GCG analysis tool, "Pile-up". For this analysis, both UMP kinases and TMP kinases were included. UMP kinases were included from archebacterial, eubacterial, and eukaryotic (plant, mammal, yeast, and mold) sources. The GenBank accessions for the sequences used in this study are as follows: *S. pombe* TMP kinase, L04126; *S. cerevisiae* TMP kinase, K02116; *H. sapiens* TMP kinase, L16991; *T. aquaticus* UMP kinase, X83598; *E. coli* UMP kinase, X78809; *S. cerevisiae* UMP kinase, M69295; *S. scrofa* UMP kinase, D29655; *D. discoideum* UMP kinase, M34568; and *A. thaliana* UMP/CMP kinase, AF000147. **Panel B.** Alignment of amino acid sequences of eukaryotic UMP kinases from a plant (*A. thaliana*), a mammal (*S. scrofa*), a yeast (*S. cerevisiae*), and a cellular slime mold (*D. discoideum*). Those residues showing complete identity among the four species are shown by bullets (•) below that residue. If conservative substitutions are allowed then additional residues are conserved (+).

Figure 3. Sequence of portions of the construct pRT380.

Panel A. A structural model of the GST-kinase fusion protein showing the amino acid sequence at the site of fusion. **Panel B.** Nucleotide and translated amino acid sequences of the pRT380 construct at the site of GST-UMP/CMP kinase fusion and the 3' end of the fusion protein. The sequences up through the *Bam*HI site are from the pGEX-4T-3 vector and encode the C-terminus of the glutathione-S-transferase. The Aspartate rich sequence extending from

the *Bam*HI site to the Methionine is the site of enterokinase recognition. Cleavage occurs after the Lysine and is indicated by the arrow. The sequence, Met-Gly-Ser-Val-Asp, are the first five amino acids of the *Arabidopsis thaliana* UMP/CMP kinase. The sequence of the remainder of the protein is identical with that presented in Figure 1 through the stop codon. After the stop codon, we introduced a second TAG stop codon and a *Xho*I site. Downstream of the *Xho*I site is the pGEX-4T-3 vector.

Figure 4. SDS polyacrylamide gel electrophoresis of UMP/CMP kinase.

All samples were analyzed on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1, cell sonicates of XL1-Blue cells containing the plasmid pRT380 with no added IPTG; Lane 2, cell sonicates of XL1-Blue cells containing the plasmid pRT380 after the addition of IPTG; Lane 3, GST-UMP/CMP kinase fusion protein eluted from glutathione-Sepharose beads; Lane 4, GST-UMP/CMP kinase fusion protein eluted from glutathione-Sepharose beads, then digested with enterokinase; Lane 5, digestion of GST-UMP/CMP kinase fusion protein with enterokinase while fusion protein is still attached to the beads; Lane 6, HPLC purified UMP/CMP kinase. Molecular weight markers used were phosphorylase b, 97.4 KDa; bovine serum albumin, 66 KDa; ovalbumin, 45k Da; carbonic anhydrase, 31 KDa; trypsin inhibitor, 21.5 KDa; and lysozyme, 14.4 KDa.

Figure 5. HPLC purification of UMP/CMP kinase.

The GST-UMP/CMP kinase fusion protein was digested with enterokinase while still attached to the glutathione-sepharose beads. The elute was applied to a 250 x 10 mm I.D. reverse phase C18 column and eluted as described in the Experimental Procedures section. Absorbance was monitored at 214 nm. The eluted protein peak containing the purified UMP/CMP kinase was collected (B=41%). The hatched area under the peak illustrates the collected pool of UMP/CMP kinase.

Figure 6. MALDI analysis of the purified UMP/CMP kinase.

Typical MALDI mass spectrum of *Arabidopsis thaliana* UMP/CMP kinase. The $[MH_2]^{2+}$ peak is at 11,286, $[MH]^+$ peak is at 22405, and $[2MH]^+$ peak is at 44984.

Figure 7. Typical sedimentation equilibrium data of the UMP/CMP kinase.

Panel A. Residuals vs. radius plot. It shows that the residuals are randomly scattered above and below the line, indicating a good fit. **Panel B.** Absorbance gradient in centrifuge cell after attaining sedimentation equilibrium. The solid curve is the result of a fit to a single ideal species. **Panel C.** Apparent molecular weight vs. protein concentration (in absorbance at 230 nm).

Figure 8. Analysis of kinetic mechanism.

Panel A. Double reciprocal plot of initial velocity versus ATP concentrations. For these experiments, the concentrations of UMP are 40 (■), 60 (+), 90 (▲), and 120 μM (□). The lines are theoretical based on Eq 1, when n = 1, and the points are experimentally determined. **Panel B.** Double reciprocal plot of initial velocity versus UMP concentrations. For these experiments, the concentrations of ATP are 15 (■), 25(+), 40(▲), and 65 mM (□). The lines are theoretical based on Eq. 1 when n = 1 and the points are experimentally determined.

Figure 9. Kinetics of AP_5A inhibition.

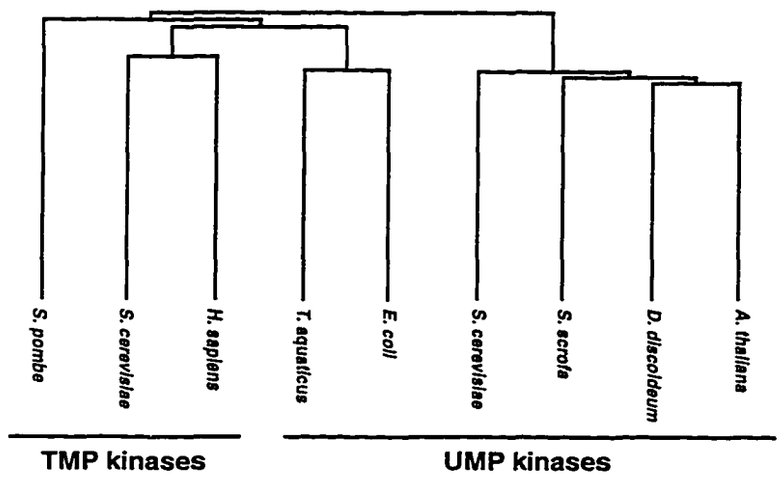
Panel A. Double reciprocal plot of initial velocity versus ATP concentrations in the presence of AP_5A inhibitor. UMP concentrations were fixed at 400 μM. The concentrations of AP_5A are: 0 (■), 2(+), 4(▲), and 6 μM (□). The lines are theoretical based on Eq. 6 and the points are experimentally determined. **Panel B.** Plot of reciprocal of initial velocity of UMP/CMP kinase versus reciprocal of UMP concentrations in the presence of AP_5A inhibitor. ATP concentrations were fixed at 300 μM. The concentration of AP_5A are 0 (■), 8 (+), 16

(▲) and 24 μM (□). The lines are theoretical based on Eq. 6 and the points are experimentally determined.

```
CCAAATTCTC TCCGTGTTTC CTTCTGCTTC AGGTGATATT TTAACAAAGT TCTTAGTACA              60

                  5                  10                  15                  20
ATG GGA TCT GTT GAT GCT GCT AAT GGA AGT GGG AAG AAA CCT ACA GTT ATA TTT GTT CTT  120
Met Gly Ser Val Asp Ala Ala Asn Gly Ser Gly Lys Lys Pro Thr Val Ile Phe Val Leu

                  25                  30                  35                  40
GGT GGT CCA GGA AGT GGA AAA GGT ACC CAG TGT GCT TAT ATT GTT GAA CAT TAT GGT TAC  180
Gly Gly Pro Gly Ser Gly Lys Gly Thr Gln Cys Ala Tyr Ile Val Glu His Tyr Gly Tyr

                  45                  50                  55                  60
ACA CAT CTG AGT GCT GGA GAT CTT CTT AGA GCT GAG ATT AAA TCA GGT TCT GAA AAT GGA  240
Thr His Leu Ser Ala Gly Asp Leu Leu Arg Ala Glu Ile Lys Ser Gly Ser Glu Asn Gly

                  65                  70                  75                  80
ACT ATG ATC CAG AAT ATG ATT AAA GAG GGG AAG ATT GTA CCT TCT GAG GTT ACT ATC AAG  300
Thr Met Ile Gln Asn Met Ile Lys Glu Gly Lys Ile Val Pro Ser Glu Val Thr Ile Lys

                  85                  90                  95                 100
CTT CTA CAG AAA GCT ATT CAG GAA AAC GGG AAT GAC AAG TTC CTC ATT GAT GGT TTC CCT  360
Leu Leu Gln Lys Ala Ile Gln Glu Asn Gly Asn Asp Lys Phe Leu Ile Asp Gly Phe Pro

                 105                 110                 115                 120
CGT AAT GAG GAA AAC CGA GCA GCA TTT GAA AAA GTT ACT GAG ATT GAA CCA AAG TTT GTC  420
Arg Asn Glu Glu Asn Arg Ala Ala Phe Glu Lys Val Thr Glu Ile Glu Pro Lys Phe Val

                 125                 130                 135                 140
TTA TTC TTC GAT TGT CCT GAG GAA GAG ATG GAG AAG CGC CTG TTG GGC CGA AAC CAG GGG  480
Leu Phe Phe Asp Cys Pro Glu Glu Glu Met Glu Lys Arg Leu Leu Gly Arg Asn Gln Gly

                 145                 150                 155                 160
AGA GAG GAT GAC AAT ATT GAG ACT ATA AGG AAG CGC TTT AAG GTG TTT CTT GAA TCT AGC  540
Arg Glu Asp Asp Asn Ile Glu Thr Ile Arg Lys Arg Phe Lys Val Phe Leu Glu Ser Ser

                 165                 170                 175                 180
TTA CCA GTG ATT CAT TAC TAC GAA GCT AAG GGG AAA GTT AGG AAG ATT AAT GCT GCA AAG  600
Leu Pro Val Ile His Tyr Tyr Glu Ala Lys Gly Lys Val Arg Lys Ile Asn Ala Ala Lys

                 185                 190                 195                 200
CCC ATT GAA GCT GTC TTC GAG GAG GTG AAG GCA ATT TTT TCT CCT GAA GCT GAG AAG GTT  660
Pro Ile Glu Ala Val Phe Glu Glu Val Lys Ala Ile Phe Ser Pro Glu Ala Glu Lys Val

    202
GAA GCC TAG                                                                   669
Glu Ala ***

     GCTGCAGCAT CGTAAAAAGA TACCGAAAAC CCGATAAATT GATCAGGTAG AGTTGGGTGT        729
     GTTGCATCTT TCTTACAGGA AGTCTCTGCT TGGCCGTATT TGTTTTTTTG CTTATTAACC        789
     ATATGATTGA TTACTGTTAT TATTATACAC AATTTACCTA TTATATAATT TTTGTTTTGT        849
     TTCAATTTTT GTATTTGATG GGATTAAAAA AAAAAAAAAA AAAAAA                       895
```

Figure 1

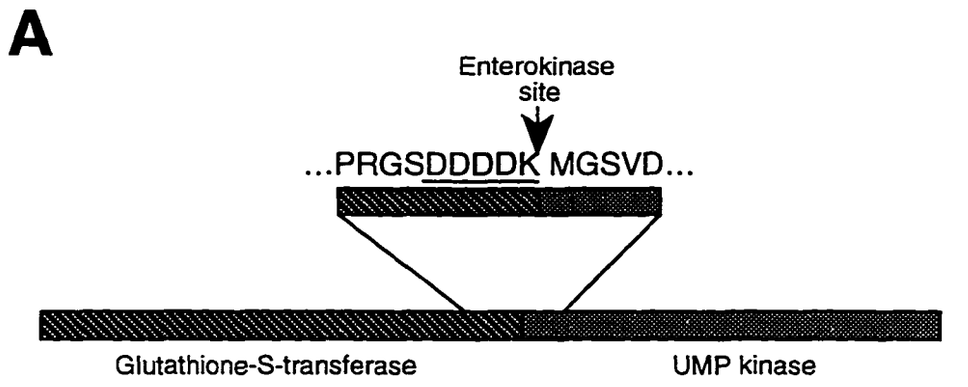
A



B

```
            1                                                    50
Arabidopsis    MGSVDAAN GSGKKPTVIF VLGGPGSGKG TQCAYIVEHY GYTHLSAGDL
Dictyostelium           MEKSKPNVVF VLGGPGSGKG TQCANIVRDF GWVHLSAGDL
Porcine                   MRPKVVF VLGGPGAGKG TQCARIVEKY GYTHLSAGEL
Yeast          MTAATTSQPA FSPDQVSVIF VLGGPGAGKG TQCEKLVKDY SFVHLSAGDL
Identity                        +++ ++++++ +++ +++  ++  +  + ++++++

            51                                                   100
Arabidopsis    LRAEIKS.GS ENGTMIQNMI KEGKIVPSEV TIKLLQKAIQ EN.....GND
Dictyostelium  LRQEQQS.GS KDGEMIATMI KNGEIVPSIV TVKLLKNAID AN.....QGK
Porcine        LRDERKNPDS QYGELIEKYI KDGKIVPVEI TISLLRREMD QTMAANAQKN
Yeast          LRAEQGRAGS QYGELIKNCI KEGQIVPQEI TLALLRNAIS DNVKAN..KH
Identity       ++ +      +   + ++   + + + +++  + +  ++   +

            101                                                  150
Arabidopsis    KFLIDGFPRN EENRAAFEKV TE..IEPKFV LFFDCPEEEM EKRLL..GRN
Dictyostelium  NFLVDGFPRN EENNNSWEEN MKDFVDTKFV LFFDCPEEVM TQRLLKRGES
Porcine        KFLIDGFPRN QDNLQGWNKT MDGKADVSFV LFFDCNNEIC IERCLERGKS
Yeast          KFLIDGFPRK MDQAISFER. ..DIVESKFI LFFDCPEDIM LERLLERGKT
Identity        +++++++   ++             ++  ++ +++++  + +   + +

            151                                                  200
Arabidopsis    QGREDDNIET IRKRFKVFLE SSLPVIHYYE AKGKVRKINA AKPIEAVFEE
Dictyostelium  SGRSDDNIES IKKRFNTFNV QTKLVIDHYN KFDKVKIIPA NRDVNEVYND
Porcine        SGRSDDNRES LEKRIQTYLQ STKPIIDLYE EMGKVKKIDA SKSVDEVFDE
Yeast          SGRSDDNIES IKKRFNTFKE TSMPVIEYFE TKSKVVRVRC DRSVEDVYKD
Identity        ++ +++ ++ ++++++  +    +  +++ +     ++          +  ++  +

            201          214
Arabidopsis    VKAIFSPEAE KVEA
Dictyostelium  VENLFKSMGF
Porcine        VVKIFDKEG
Yeast          VQDAIRDSL
Identity       +  ++
```

Figure 2


A
Enterokinase
site
...PRGSDDDDK MGSVD...
Glutathione-S-transferase
UMP kinase

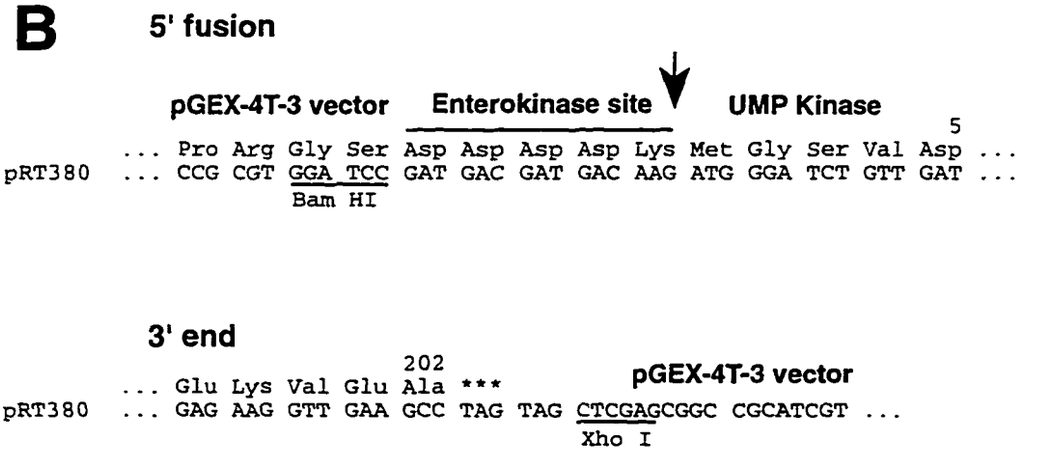

B
5' fusion
pGEX-4T-3 vector
Enterokinase site
UMP Kinase
5
... Pro Arg Gly Ser Asp Asp Asp Asp Lys Met Gly Ser Val Asp ...
pRT380 ... CCG CGT GGA TCC GAT GAC GAT GAC AAG ATG GGA TCT GTT GAT ...
Bam HI
3' end
202
... Glu Lys Val Glu Ala *** pGEX-4T-3 vector
pRT380 ... GAG AAG GTT GAA GCC TAG TAG CTCGAGCGGC CGCATCGT ...
Xho I

Figure 3

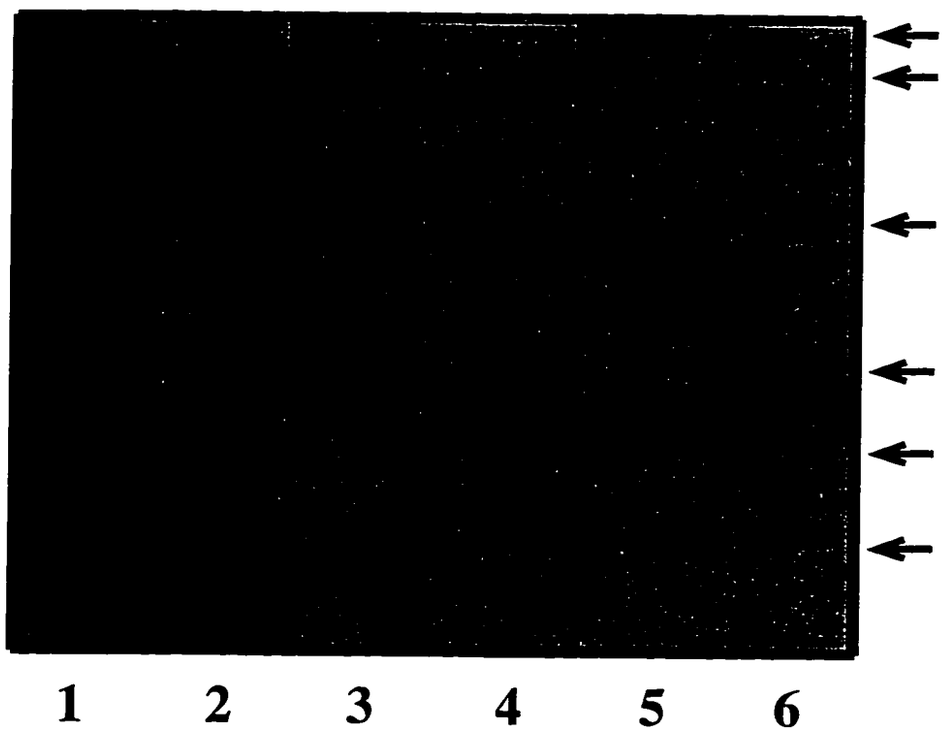


Figure 4

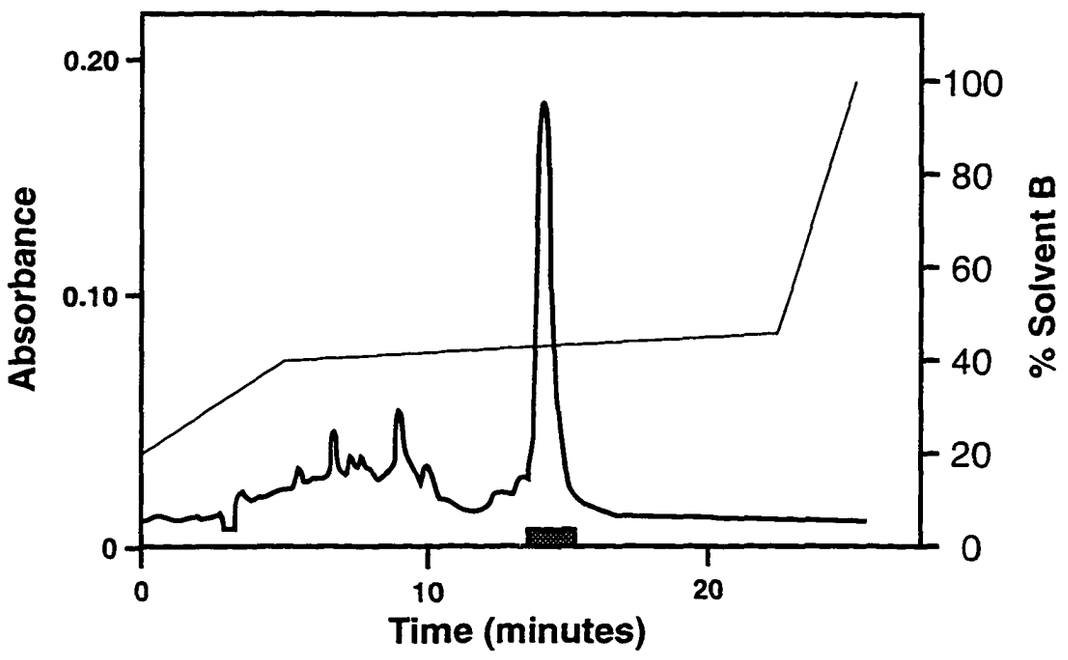

0.20
0.10
0
Absorbance
100
80
60
40
20
0
% Solvent B
0
10
20
Time (minutes)

Figure 5

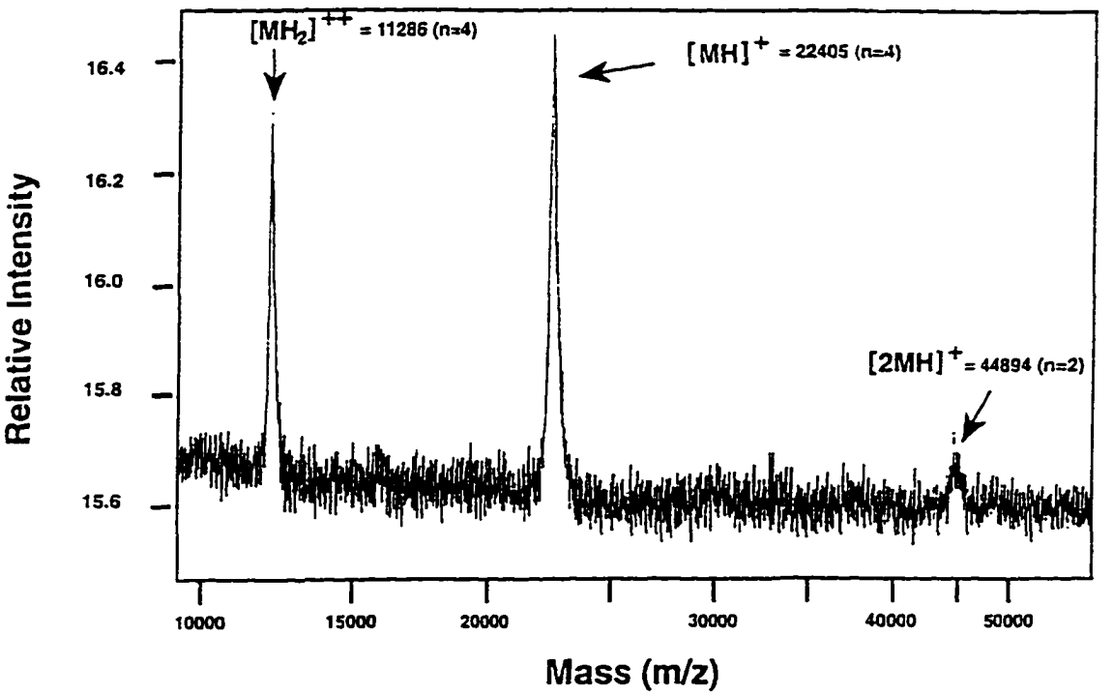

[MH2]++ = 11286 (n=4)
[MH]+ = 22405 (n=4)
[2MH]+ = 44894 (n=2)
Relative Intensity
16.4
16.2
16.0
15.8
15.6
10000
15000
20000
30000
40000
50000
Mass (m/z)

Figure 6

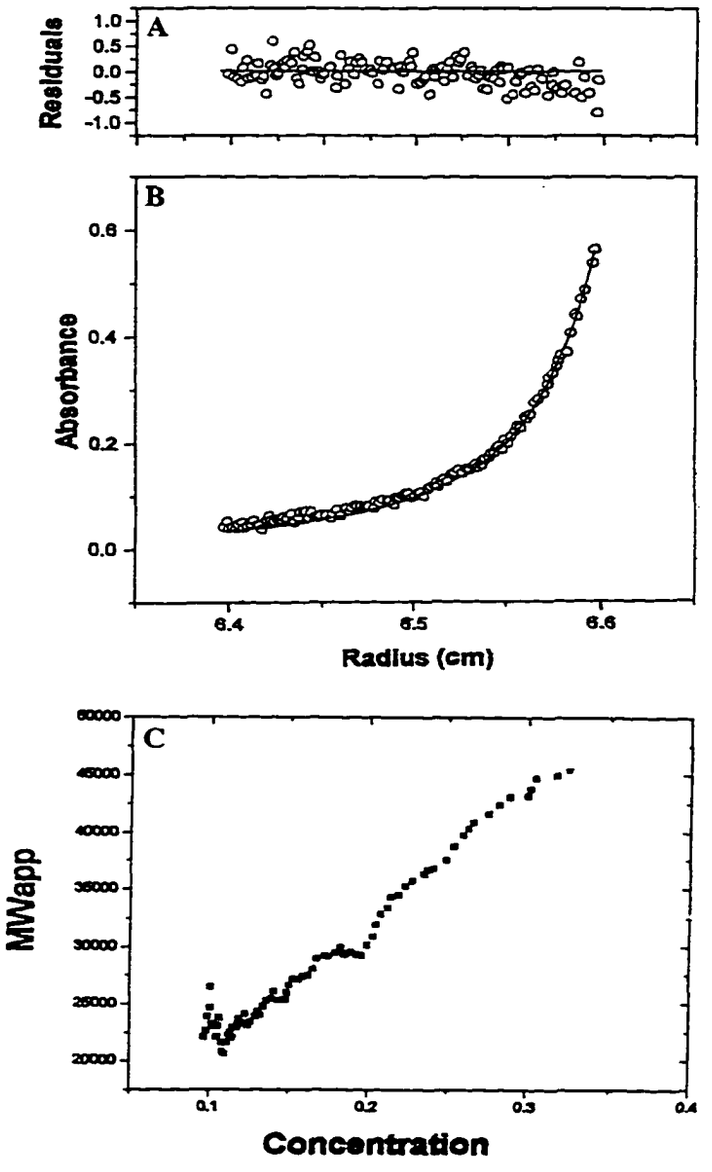

A
Residuals
1.0
0.5
0.0
-0.5
-1.0
B
Absorbance
0.8
0.4
0.2
0.0
6.4
6.5
6.6
Radius (cm)
C
MWapp
60000
50000
40000
30000
20000
0.1
0.2
0.3
0.4
Concentration

Figure 7

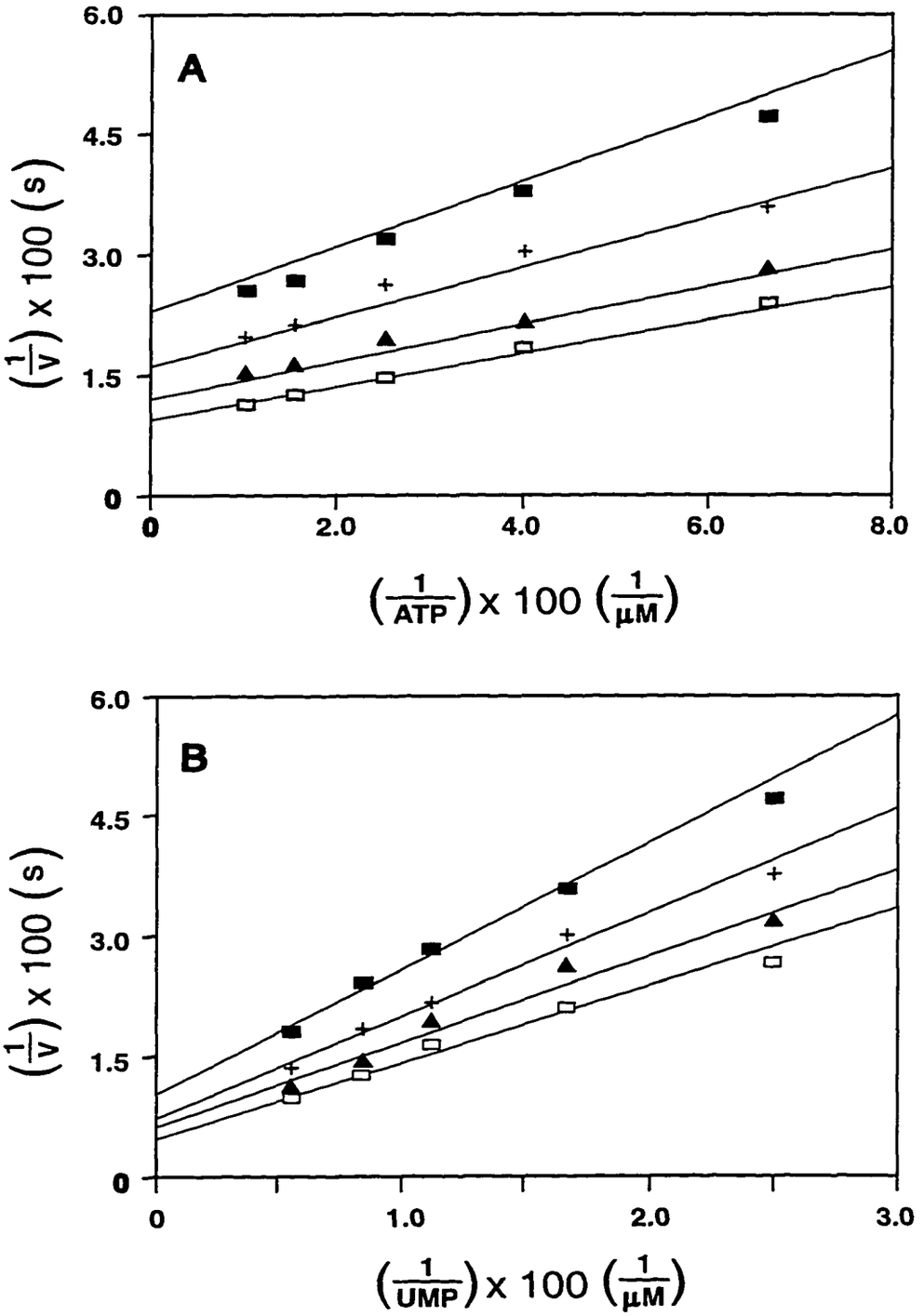

A
6.0
4.5
3.0
1.5
0
0
2.0
4.0
6.0
8.0
(1/V) x 100 (s)
(1/ATP) x 100 (1/µM)
B
6.0
4.5
3.0
1.5
0
0
1.0
2.0
3.0
(1/V) x 100 (s)
(1/UMP) x 100 (1/µM)

Figure 8

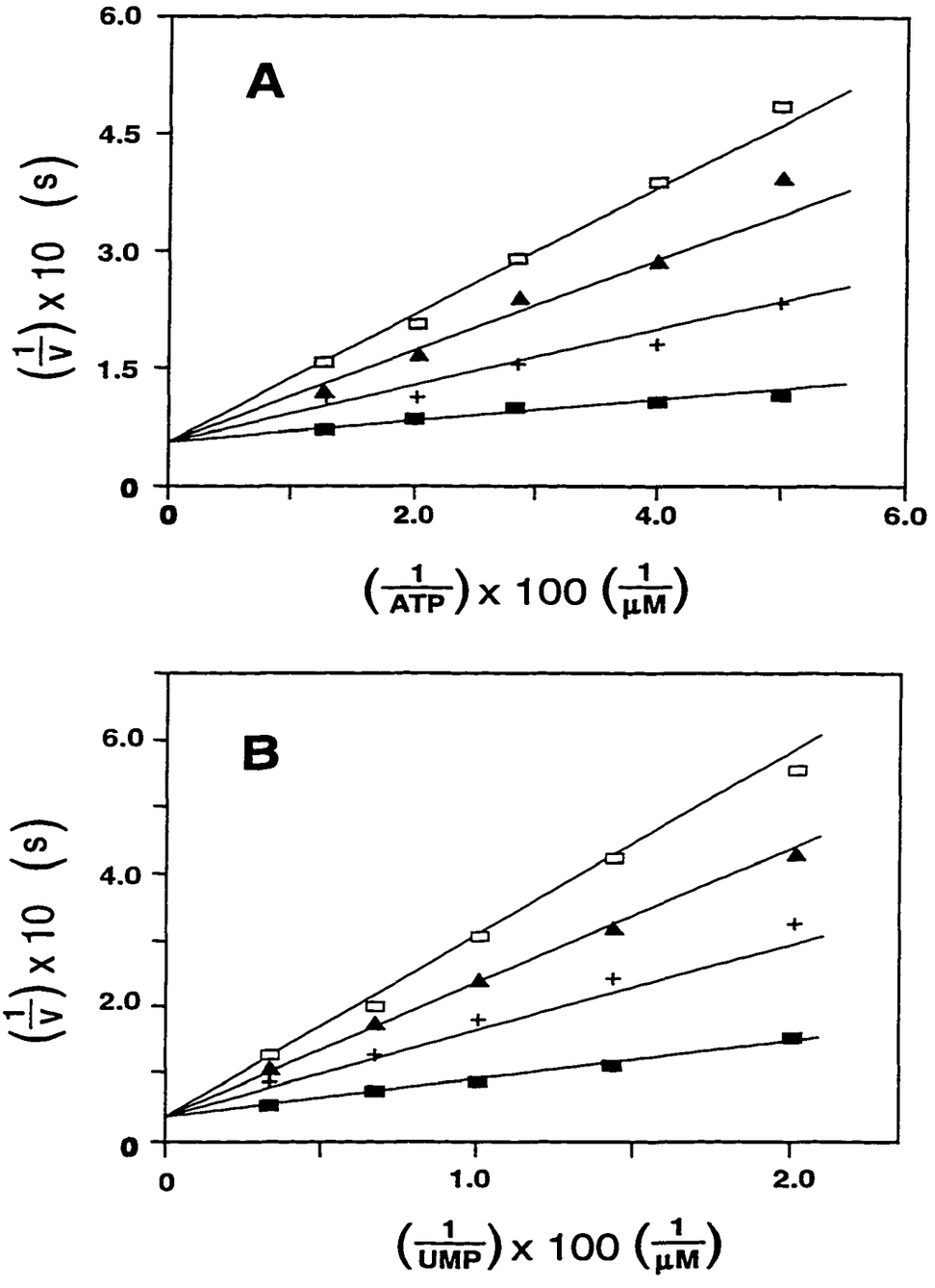

A
6.0
4.5
3.0
1.5
0
0
2.0
4.0
6.0
$(\frac{1}{v}) \times 10$ (s)
$(\frac{1}{ATP}) \times 100\ (\frac{1}{\mu M})$
B
6.0
4.0
2.0
0
0
1.0
2.0
$(\frac{1}{v}) \times 10$ (s)
$(\frac{1}{UMP}) \times 100\ (\frac{1}{\mu M})$

Figure 9

CHAPTER 5. EFFECT OF MUTATIONS OF CONSERVED RESIDUES IN THE PHOSPHATE-BINDING SEQUENCE IN *ARABIDOPSIS THALIANA*

A paper to be submitted to the *Arch. Biochem. Biophys.*

Lan Zhou[1] and Robert W. Thornburg[1,2]

Abstract

All eukaryotic UMP/CMP kinases contain a glycine-rich sequence GGPG(S/A)GK at the N-terminus. This sequence is homologous to the conserved sequence GXXGXGK found in other ATP-binding proteins. To study the role of this conserved sequence in *Arabidopsis* UMP/CMP kinase, five conserved residues were mutated by site-directed mutagenesis to generate seven mutant enzymes: G21A, G22A, G24A, G26A, K27R, K27M and K27E. The G21A and G26A mutants were unable to be purified due to degradation during the purification phase. Kinetic studies on the rest of mutants when compared to studies on the wild-type enzyme revealed that this sequence is important for ATP-binding and enzyme catalysis. All the mutants had a decreased k_{cat}/K_m value for ATP. The G22A and G24A mutants had about a half of the k_{cat} value of wild-type and 3.9-fold and 3.3-fold increase in K_m values, respectively. The k_{cat}/K_m values for ATP in the K27M and K27E mutants were changed significantly and increased by 1000-fold and 2600-fold, respectively. The removal of the terminal positive charge of Lys27 in the K27M and K27E mutants resulted in 20% of the k_{cat} value of wild-type. However, both mutants had a remarkable increase in K_m value for ATP by 228-fold and 537-fold, respectively. Therefore, the positive charge of Lys27 plays an important role on both ATP binding and enzyme catalysis. Interestingly, the results also showed that the mutations which affected ATP binding also had an effect on UMP binding.

---

*This work was sponsored by a grant (91-37301-6208) from the US Department of Agriculture.

[1]Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

[2]To whom correspondence should be addressed.

Introduction

Adenine nucleotide-binding proteins are involved in many biological processes such as membrane transport, cell division, protein export and DNA repair (Higgins et al., 1986; 1988). These proteins share an unique structural feature among them: a glycine-rich phosphate-binding loop (P-loop), GXXGXGK (X represents an unspecified amino acid residue.), is found in the amino acid sequence near the N-terminus of the protein (Walker et al., 1982; Higgins et al., 1988). The conserved P-loop is thought to be important for nucleotide binding (Pai et al 1990) and forms a large anion hole which accommodates phosphates of ATP (Dreusicke and Schulz, 1986).

UMP/CMP kinase catalyzes the transfer of phosphoryl group from ATP to either UMP or CMP to form ADP and UDP or CDP. All pyrimidines within the cell are derived from UMP. Therefore, UMP kinase catalyzes the first committed step of pyrimidine metabolism. The genes or cDNAs encoding UMP kinase have been cloned from *E. coli* (Serina et al., 1995), *D. discoideum* (Wiesmüller et al., 1990), *S. cerevisiae* (Liljelund and Lacroute, 1986), *S. scrofa* (Okajima et al., 1995) and *A. thaliana* (Zhou et al., submitted). Alignment of the amino acid sequences of all four eukaryotic UMP kinases reveals that a glycine-rich sequence, GGPG(S/A)GK found in the N-terminal region, is conserved in all the enzymes.

What are the functions of this P-loop? The crystal structures of UMP kinases from *S. cerevisiae* and *D. discoideum* have been solved (Müller-Dieckmann and Schulz 1994 and 1995; Scheffzek et al., 1996). The crystal structure of UMP/CMP kinase from *D. discoideum* complexed with a bisubstrate inhibitor P^1-(5'-adenosyl) P^5-(5'-uridyl) pentaphosphate (UP_5A) with a resolution of 2.2 Å showed that the globular enzyme consisted of eight α-helixes that surround five parallel β-sheets. The P-loop was located between a β-sheet (β1) and an a-helix (α2). The residues of the P-loop (Gly13, Gly14, Gly16 and Lys19) with the conserved arginines formed interactions with the five phosphoryl groups of UP_5A in the active center of the enzyme. The structure also showed that the NH_3 group of Lys19 in the P-loop interacted

strongly with oxygens of P2 (corresponding to β phosphoryl group of ATP) and P4 of UP_5A and is stabilized additionally by the main-chain carbonyl group of Gly14. The Lys19 together with Mg^{2+} is responsible for the positioning of the reacting phosphoryl groups. The similar situation was found for the P-loop in the yeast enzyme. Therefore, the residues in the P-loop, especially Lys19, are important in ATP binding and/or enzyme catalysis. Any change in the conserved residues may result in alterations in the folding pattern and nucleotide binding (Bourne et al., 1990 and 1991; Saraste et al., 1990).

In order to study the structure and function relationship of *Arabidopsis* UMP/CMP kinase, we chose to study the phosphate-binding sequence of ATP by site-directed mutagenesis. We have made several mutations in the P-loop of the *Arabidopsis* UMP/CMP kinase. The kinetic studies showed this P-loop is important for ATP binding and enzyme activity as predicted from the available crystal structures from other sources.

Experimental Procedures

Materials

The pGEX-4T-3 and glutathione Sepharose 4B were from Pharmacia (Pharmacia Biotech, Piscataway, NJ). The vector pBluescript II SK (+/-) and *Pfu* DNA polymerase were from STRATAGENE (La Jolla, CA). Enterokinase was from Biozyme Laboratories (San Diego, CA). Restriction enzymes and T4 DNA Ligase were from Promega (Madison, WI). Oligonucleotides were synthesized at the Nucleic Acid Facility of Iowa State University. All other enzymes and reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. The bacterial strain XL1-BLue was used for all bacterial manipulations and expression.

Site-directed mutagenesis by PCR and construction of mutant plasmids

Single-site mutations of amino acid residues were introduced by two-rounds of PCR according to the method of Ausubel et al (1993). Figure 1 summarizes the strategy used to generate the mutation. All the primers used are listed in Table 1. Primer P1 was designed based on the sequence of the vector pGEX-4T-3. Primer P2 and P3 contained a specific

mutation for one mutant. Primer P4 was based on the sequence of the vector pRT380. This primer also contained a *Xho*I site and an additional stop codon. The plasmid pRT380 was used as a template for all the mutations in the first-round PCR. The plasmid pRT380 was constructed by inserting the 637 bp *Bam*HI/*Xho*I fragment containing the intact *Arabidopsis* UMP/CMP kinase open reading frame, an enterokinase site for cleavage of the GST fusion protein and an additional stop codon into the *Bam*HI/*Xho*I sites of pGEX-4T-3. The first-round PCR was performed in two separate reactions using two sets of primers P1 and P3 or P2 and p4. Depending on which PCR oligonucleotides were used for the different mutants, PCR products of about 320 bp and 560 bp were gel-purified and used as templates in the second-round PCR using primer P1 and P4. The 880 bp PCR product was digested with *Bam*HI and *Xho*I. The resulting 637 bp fragment was subcloned into pBluescript II SK (+/-) and the mutation was confirmed by sequencing using T3 and T7 primers. The 637 bp *Bam*HI/*Xho*I fragment then was excised from pBluescript II SK (+/-) and subcloned into pGEX-4T-3. Each mutant cDNA construct was transformed to bacterial strain XL1-BLue and confirmed by sequencing using primers on the vector. The clones which had been verified by sequencing were further used for the expression and purification of the corresponding mutants.

Purification and kinetic studies of wild-type and mutant UMP/CMP Kinases

The protocols for expression and purification of the mutant UMP/CMP kinases were the same as those for the wild-type enzyme (Zhou et al., submitted). Protein purity was analyzed by SDS PAGE according to the method of Laemmli (1970). Protein concentration was determined by Bradford assay with bovine serum albumin as a standard (Bradford, 1976). UMP kinase activity was determined as described previously (Zhou et al., submitted). K_m values for each substrate were obtained by keeping the other substrate at the saturating level (3-10 times of its K_m value) and varying the concentration of the test substrate. Substrate inhibition was not found with any of the enzymes tested. All kinetic data were analyzed by using the computer program ENZFITTER (Leatherbarrow, 1987). The V_{max} value used for

calculating k_{cat} value was the average of five initial rates at saturating concentrations of both ATP and UMP substrates.

Matrix-assisted laser desorption ionization (MALDI) mass spectroscopy

Protein samples of 0.5 to 1.0 µl containing about 0.5 to 1 µg of protein, were loaded with 0.5 µl of freshly prepared 3,5-dimethoxy-4-hydroxy cinnamic acid matrix into a Finnigan LASERMAT 2000 MALDI-time of flight mass analyzer. The collected data were analyzed using the LASERMAT 2000 data processing software. Lysozyme was used as an internal calibration standard.

Circular dichroism spectroscopy

Circular dichroism spectroscopic (CD) studies on the wild-type and mutant forms of UMP/CMP kinase were performed in 20 mM MES buffer (pH 6.5) at room temperature in a JASCO CD Model J-710 spectrometer. Samples were placed in a 1-mm cuvette and data points were collected from 190 to 260 nm in 0.5 nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed by using the Standard Analysis program provided with the spectrometer.

Results

Mutagenesis of UMP/CMP kinase cDNA

The alignment of amino acid sequences showed the comparison of the ATP-binding consensus sequence GXXGXGK and the corresponding regions of the UMP kinases from four different eukaryotes. The alignment of amino acid sequences of these UMP kinases showed that all the enzymes contain a conserved sequence GGPG(S/A)GK at the N-terminus of the protein, which is homologous to the conserved sequence GXXGXGK found in many other ATP-binding proteins (Higgins et al 1988). This suggested that this glycine-rich sequence in *Arabidopsis* UMP/CMP kinase may play as important a role in ATP binding as it does in other ATP-binding proteins. Therefore, site-directed mutagenesis was conducted to introduce a series of the mutations within this consensus sequence of *Arabidopsis* UMP/CMP

kinase. The conserved glycine residues at position 21, 24 and 26 were changed to alanine. The glycine residue at position 22 was conserved among all four eukaryotic UMP kinases. Therefore, it was also mutated to alanine. The conserved lysine at position 27 was changed to arginine, methionine or glutamic acid. The arginine mutant (K27R) was prepared to retain the terminal positive charge. The methionine mutant (K27M) was designed to remove the terminal positive charge of lysine while maintaining the long alkyl side chain. The glutamic acid mutant (K27E) was used to remove the terminal positive charge and introduce a negative charge. Therefore, these three mutants at the same position were expected to interpret any differences in kinetic data caused by change of size or charge of the lysine residue.

Purification of the mutant enzymes

The procedures used to purify the mutant enzymes were basically the same as that for the wild-type enzyme (Zhou et al., submitted). Five of the mutant enzymes were successfully purified. The purity was detected on SDS PAGE. Both the mutant and wild-type enzymes showed the single bands (about 22 KDa) and had the same migration distance on the gel (Figure 2). MALDI analysis verified that the molecular mass of all the mutant enzymes was the same as that of wild-type enzyme (data not shown). However, the G21A and G26A mutants could not be purified. In contrast to the other mutant proteins, both of these mutant fusion proteins were unstable and degraded during the purification process.

Secondary structure analysis

The secondary structures of the wild-type and mutant enzymes were analyzed by CD spectrometry. This CD study was used to check whether localized or global structural alterations were introduced by the various mutations. The CD spectral results indicated that the spectrum of each mutant was essentially superimposable on that of the wild-type enzyme (data not shown). Therefore, these CD results suggested that no major conformational changes occurred in any of the mutants compared to the wild-type enzyme.

Kinetic analysis of UMP/CMP kinase mutants

To determine the effect of the introduced mutations on substrate binding and enzyme activity, initial rate kinetic studies of the mutant UMP/CMP kinases were performed and the data were compared with those of wild-type enzyme. The results, summarized in Table 2, showed that each of the mutations caused a decrease in the k_{cat} value compared to the wild-type enzyme. The k_{cat} values for the G22A and G24A mutants were 4.51 (s^{-1}) and 3.69 (s^{-1}), respectively, which are equivalent to about 50% of the wild type activity (7.64 (s^{-1})). Therefore, the mutations at position 22 and 24 have a modest effect on enzyme catalysis. The greatest changes in the k_{cat} values were observed in the mutations at position 27. The k_{cat} values for K27R, K27M and K27E were about 47%, 23% and 21% of the k_{cat} value of the wild-type activity, respectively.

In contrast to the k_{cat} values, the K_m values for ATP for all the mutants except the K27R mutant increased. The K_m values for the G22A and G24A mutants were 3.9-fold and 3.3-fold greater than that of wild-type enzyme. The K_m values for ATP for the K27M and K27E mutants showed the greatest changes. These K_m values increased by 228-fold and 537-fold, respectively. On the other hand, almost no change in the K_m value was observed for the K27R mutant.

Interestingly, all the mutations except the arginine substitution at position 27 also had an effect on the K_m value for UMP. The K_m values for the G22A and G24A mutants increase by 2.5-fold and 13.7-fold, respectively. However, the K_m values for the K27M and K27E mutants decreased by 8.6-fold and 26.4-fold, respectively. The K_m value for the K27R mutant had almost no change.

Discussion

Arabidopsis UMP/CMP kinase, like other eukaryotic UMP kinases and many other ATP-binding proteins, contains a conserved glycine-rich sequence near the N-terminus of the polypeptide chain. The crystal structures of UMP kinases from yeast and *D. discoideum*

suggested that this consensus sequence may play a role in ATP binding and/or enzyme catalysis (Müller-Deickmann and Schulz, 1994 and 1995; Scheffzek et al., 1996). The site-directed mutagenesis studies in this report support this suggestion. Substitutions of glycine residues with alanine at position 22 and 24 and lysine at position 27 with arginine, methionine or glutamic acid all resulted in a decrease in the k_{cat}/K_m value for substrate ATP. The greatest changes in the k_{cat}/K_m values were observed in the K27M and K27E mutants with decrease by 1000-fold and 2600-fold, respectively. These results strongly suggest that the residues in the P-loop, especially lysine, are important in ATP binding and enzyme activity. The results from the three mutations at position 27 indicated that this lysine residue is critical for ATP binding and enzyme catalysis. The substitution of lysine with arginine to retain the positive charge resulted in an enzyme that had a k_{cat} value of about half of that of the wild-type enzyme. The k_{cat}/K_m value for ATP was 60% of that of wild-type enzyme. Because the K27R mutant retained the positive charge of the side chain, the decrease in the k_{cat} value of the K27R mutant must arise from steric differences in the respective side chains of the lysine and arginine. When the positive charge of this side chain was removed in the K27M mutant, the k_{cat} value was reduced to 23% of that of wild-type enzyme. The K_m value for ATP increased by 228-fold and the k_{cat}/K_m value for ATP was reduced by 1000-fold. Similar results were observed in the K27E mutant. These data suggest that the terminal positive charge of Lys27 is very important for ATP binding. The substitution of this positive charge with an uncharged or negative charged residue resulted in a significant decrease in ATP binding for the enzyme. The negative charge substitution in the K27E mutant made ATP binding more difficult than non-charge substitution in the K27M mutant. The results also indicated that the structure of the side chain of Lys27 had less effect on ATP binding compared to the positive charge on the side chain. Of those amino acids examined in this study, the Lys27 is much more important for the enzyme to bind ATP.

Unfortunately, two mutants with substitutions of conserved glycine residues at position 21 and 26 by alanine were unable to be purified for these studies due to degradation. However, this indicated that both two glycine residues may be essential to maintain the correct conformation of the protein and function of *Arabidopsis* UMP/CMP kinase.

It's interesting to note that the mutations which had an effect on ATP binding also had an effect on UMP binding. It seems that the binding of one substrate affects the affinity of these mutant enzymes for the other substrate. The similar case has been found in CMP kinase from *E. coli* (Bucurenci et al 1996). The *E coli* CMP kinase shows little overall sequence similarities with other known NMP kinases, but it contained the conserved sequences involved in substrate binding and catalysis. An unique feature not previously observed was that binding of CMP enhanced the affinity of the enzyme for ATP. Further experiments to show the molecular basis of substrate binding from the 3-D structure and function studies of *Arabidopsis* UMP/CMP kinase will be needed to confirm the hypothesis. In the case of *Arabidopsis* UMP/CMP kinase, the binding of two substrates to the enzyme is random (Zhou et al., submitted), which means that binding of ATP or UMP to the enzyme is not affected by binding of the other substrate. This random mechanism does not contradict with the above hypothesis.

Guanine nucleotide-binding proteins, like adenine nucleotide-binding proteins, contain a glycine-rich consensus sequence GXXXXGK. In addition, these proteins contain two other consensus sequences which do not exist in *Arabidopsis* UMP/CMP kinase: a four-nucleotide phosphate-binding sequence (D/E)XX(G/A) and a guanine-specific binding region (N/T/Q)KXD (Bourne et al., 1990 and 1991; Saraste et al., 1990). Substrate specificity studies on *Arabidopsis* UMP/CMP kinase showed that GTP was a poor phosphate donor. The enzyme using GTP and UMP as substrates only had 5% of the activity when ATP was a phosphate donor (Zhou et al., submitted). This indicated that the P-loop found both in ATP- and GTP-binding proteins is not sufficient in itself to account for GTP binding. Other conserved sequences are also necessary. In fact, in the structure of the enzyme from yeast

(Müller-Dieckmann and Schulz, 1994 and 1995) or *D. discoideum* (Scheffzek et al., 1996), there were many other conserved residues involved in ATP-binding in the active site of the enzyme. It may be oversimplified to emphasize that the P-loop alone controls the overall structure of ATP-binding site.

In this P-loop study by site-directed mutagenesis, we have found that the P-loop in *Arabidopsis* UMP/CMP kinase plays a role in ATP-binding and enzyme catalysis. It would be interesting to see how the conserved residues in the P-loop interact with the phosphoryl groups of ATP in the 3-D structure of the enzyme. This would provide structural basis for the alterations of kinetic parameters by the site-directed mutagenesis in this report. The 3-D structure also would provide insight into how the base specificity is determined, which would be a guide to change substrate specificity by site-directed mutagenesis.

Acknowledgments

We would like to thank Dr. Herbert Fromm for helpful discussion on site-directed mutagenesis.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1993) Current Protocols in Molecular Biology. John Wiley & Sons, New York, pp.8.5.1-8.5.10.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72, 248-254

Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348, 125-132.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. Nature 349, 117-127.

Bucurenci, N., Sakamoto, H., Briozzo, P., Palibroda, N., Serina, L., Sarfati, R.S., Labesse, G., Briand, G., Danchin, A., Bârzu, O. and Gilles, A.M. (1996) CMP kinase from *Escherichia coli* is structurally related to other nucleoside monophosphate kinases. J. Biol. Chem. 271, 2856-2862.

Dreusicke, D. and Schulz, G.E. (1986) The glycine-rich loop of adenylate kinase forms a giant anion hole. FEBS Letter 208, 301-304.

Higgins, C.F., Hiles, I.D., Salmond, G.P.C., Gill, D.R., Downie, J.A., Evans, I.J., Holland, B., Gray, L., Buckel, S.D., Bell, A.W. and Hermodson, M.A. (1986) A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature 323, 448-450.

Higgins, C.F., Gallagher, M.P., Mimmack, M.L. and Pearce, S.R. (1988) A family of closely related ATP-binding subunits from prokaryotic and eukaryotic cells. BioEssays 8, 111-116.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Leatherbarrow, R.J. (1987) ENZFITTER, A non-linear regression data analysis program for the IBM PC, p. 13-75, Elsevier Science Publishers BV, Cambridge, United Kingdom.

Liljelund, P. and Lacroute, F. (1986) Genetic characterization and isolation of the *Saccharomyces cerevisiae* gene coding for uridine monophosphokinase. Mol. Gen. Genet. 205, 74-81.

Müller-Dieckmann, H.J. and Schulz, G.E. (1994) The structure of uridylate kinase with its substrates, showing the transition state geometry. J. Mol. Biol. 236, 361-367.

Müller-Dieckmann, H.J. and Schulz, G.E.(1995) Substrate specificity and assembly of the catalytic center derived from two structures of ligated uridylate kinase. J. Mol. Biol. 246, 522-530.

Okajima, T., Goto, S., Tanizawa, K., Tagaya, M., Fukui, T., Shimofuruya, H. and Suzuki, J. (1995) Cloning, sequencing, and expression in *Escherichia coli* of cDNA encoding porcine brain UMP-CMP kinase. J. Biochem. 117, 980-986.

Pai, E.F., Krengel, U., Petsko, G.A., Goody, R.S., Kabsch, W. and Wittinghofer, A. (1990) Refined crystal structure of the triphosphate confirmation of H-*ras* p^{21} at 1.35 Å resolution: implications for the mechanism of GTP hydrolysis. EMBO J. 9, 2351-2359.

Saraste, M., Sibbald, P.R. and Wittinghofer, A. (1990) The P-loop - a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. 15, 430-434.

Scheffzek, K., Kliche, W., Wiesmüller, L. and Reinstein, J. (1996) Crystal structure of the complex of UMP/CMP kinase from *Dictyostelium discoideum* and the bisubstrate inhibitor P^1-(5'-adenosyl) P^5-(5'-uridyl) pentaphosphate (UP_5A) and Mg^{2+} at 2.2 Å: implications for water-mediated specificity. Biochem. 35, 9716-9727.

Serina, L., Blondin, C., Krin, E., Sismeiro, O., Danchin, A., Sakamoto, H., Gilles, A.M. and Bârzu, O. (1995) *Escherichia coli* UMP-kinase, a member of the aspartokinase family, is a hexamer regulated by guanine nuleotides and UTP. Biochemistry 34, 5066-5074.

Walker, J.E., Saraste M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the α-and β-subunits of ATP synthase, myosin, kinase and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 8, 945-951.

Wiesmüller, L., Noegel. A.A., Bârzu, O., Gerisch, G. and Schleicher, M. (1990) cDNA-derived sequence of UMP-CMP kinase from *Dictyostelium discoideum* and expression of the enzyme in *Escherichia coli*. J. Biol. Chem. 265, 6339-6345.

Zhou, L., Lacroute, F. and Thornburg, R. W. Cloning, Expression in *E. coli* and characterization of *Arabidopsis thaliana* UMP/CMP kinase. (submitted)

Table 1 Primers used in PCR to generate mutant *Arabidopsis* UMP/CMP kinase cDNAs.

Internal primers	P2	P3
G21A	5'-GTTATATTTGTTCTTGCTGGTCCA-3'	5'-GCAAGAACAAATATAACTGTAGGT-3'
G22A	5'-GTTCTTGGTGCTCCAGGAAGTGGA-3'	5'-CTGGAGCACCAAGAACAAATATAA-3'
G24A	5'-GGTGGTCCAGCAAGTGGAAAAGGT-3'	5'-CACTTGCTGGACCACCAAGAACAA-3'
G26A	5'-CCAGGAAGTGCAAAAGGTACCCAG-3'	5'-CTTTTGCACTTCCTGGACCACCAA-3'
K27R	5'-GGAAGTGGAAGAGGTACCCAGTGT-3'	5'-GTACCTCTTCCACTTCCTGGACCA-3'
K27M	5'-GGAAGTGGAATGGGTACCCAGTGT-3'	5'-GTACCCATTCCACTTCCTGGACCA-3'
K27E	5'-GGAAGTGGAGAAGGTACCCAGTGT-3'	5'-GTACCTTCTCCACTTCCTGGACCA-3'

External primers	P1	P4
	5'-GGTGATCATGTAACCCATCCTG-3'	5'-CGGCTCGAGCTACTAGGCTTCAACCTTCTCAGC-3'

Table 2 Kinetic parameters of wild-type and mutant UMP/CMP kinases from *Arabidopsis thaliana*[a]

Protein	k_{cat} (s^{-1})	K_m^{ATP} (μM)	K_m^{UMP} (μM)	k_{cat}/K_m^{ATP} 10^{-2} (s μM)$^{-1}$	k_{cat}/K_m^{UMP} 10^{-2} (s μM)$^{-1}$
Wild-type	7.64 ± 0.24	29.4 ± 2.8	152.9 ± 14.5	25.98 ± 0.26	5.00 ± 0.04
G22A	4.51 ± 0.09	114.6 ± 8.3	382.4 ± 33.7	3.94 ± 0.02	1.17 ± 0.01
G24A	3.69 ± 0.03	95.7 ± 8.5	(2.1 ± 0.6)10^3	3.86 ± 0.03	0.14 ± 0.01
K27R	3.41 ± 0.30	22.1 ± 3.7	133.0 ± 7.1	15.43 ± 0.55	2.56 ± 0.03
K27M	1.73 ± 0.10	(6.7 ± 0.9)10^3	17.7 ± 1.8	(2.58 ± 0.06)10^{-2}	9.77 ± 0.13
K27E	1.57 ± 0.02	(15.8 ± 1.4)10^3	5.8 ± 1.2	(0.99 ± 0.01)10^{-2}	27.07 ± 1.16

[a]The standard enzyme reaction contained 50 mM MES, pH 6.5, 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenoylpyruvate, 0.2 mM NADH, nucleotides, 3.5 units of pyruvate kinase and 5 units of lactate dehydrogenase in a final volume of 1 ml. When one substrate was used as the variable substrate, the other substrate was fixed at the saturating concentration. The reaction was started by addition of UMP/CMP kinase. The change in optical density at 340 nm was recorded. One unit of UMP/CMP kinase is defined as the amount of enzyme that catalyzed the formation of 1 μmole of UDP or CDP per min.

Figure Legends

Figure 1. Schematic diagram of site-directed mutagenesis by PCR.

The P1, P2, P3 and P4 represent the four primers used in PCR. The pRT380 is the expression construct containing the *Arabidopsis* wild-type UMP/CMP kinase cDNA. The specific mutation site is shown as *.

Figure 2. SDS PAGE analysis of purified wild-type and mutant UMP/CMP kinases.

All samples were run on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1, wild-type; Lane 2, G22A; Lane 3, G24A; Lane 4, K27R; Lane 5, K27M; Lane 6, K27E; Lane 7, molecular weight markers.

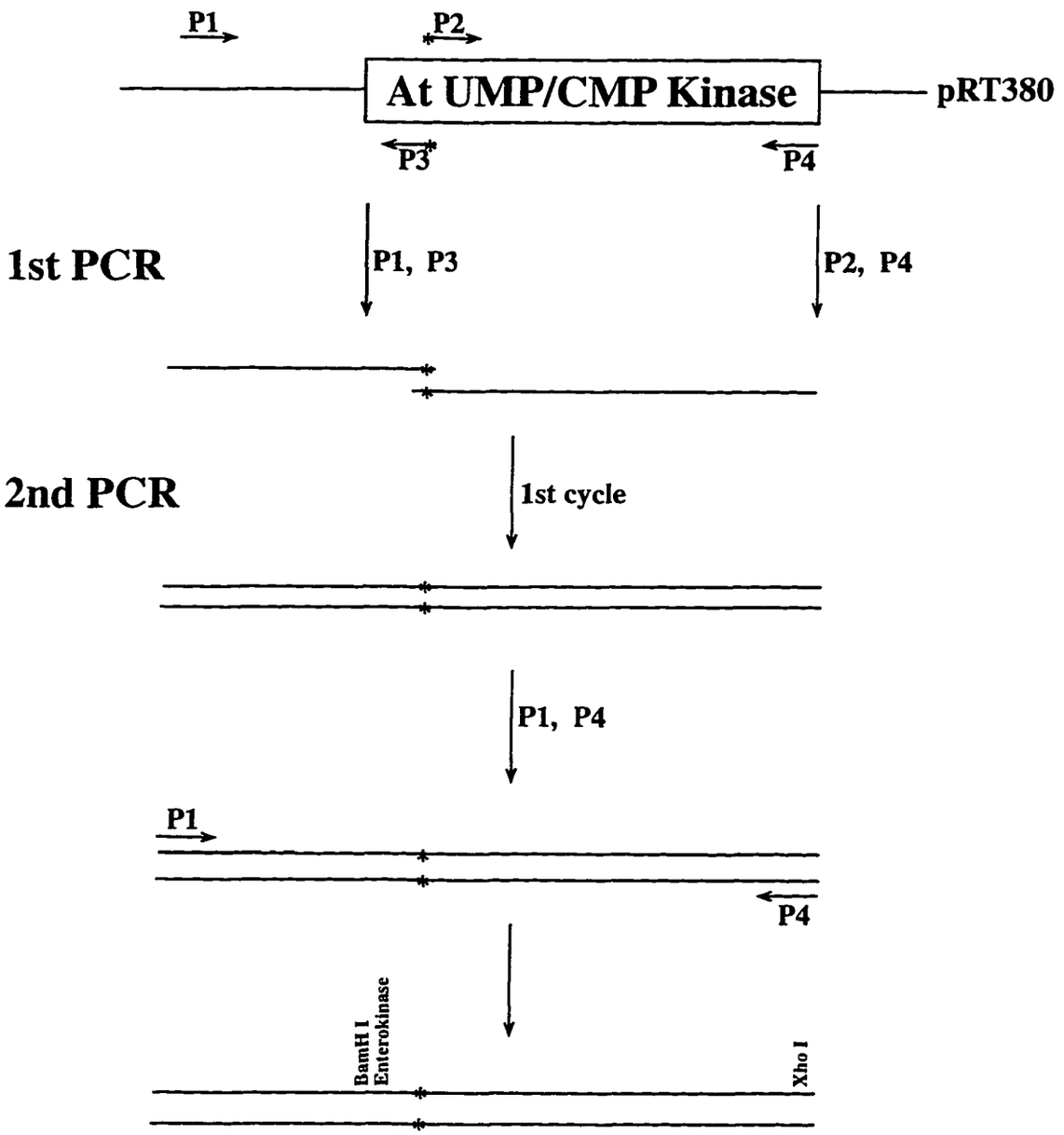

P1
P2
At UMP/CMP Kinase
pRT380
P3
P4
1st PCR
P1, P3
P2, P4
2nd PCR
1st cycle
P1, P4
P1
P4
BamH I
Enterokinase
Xho I

Figure 1

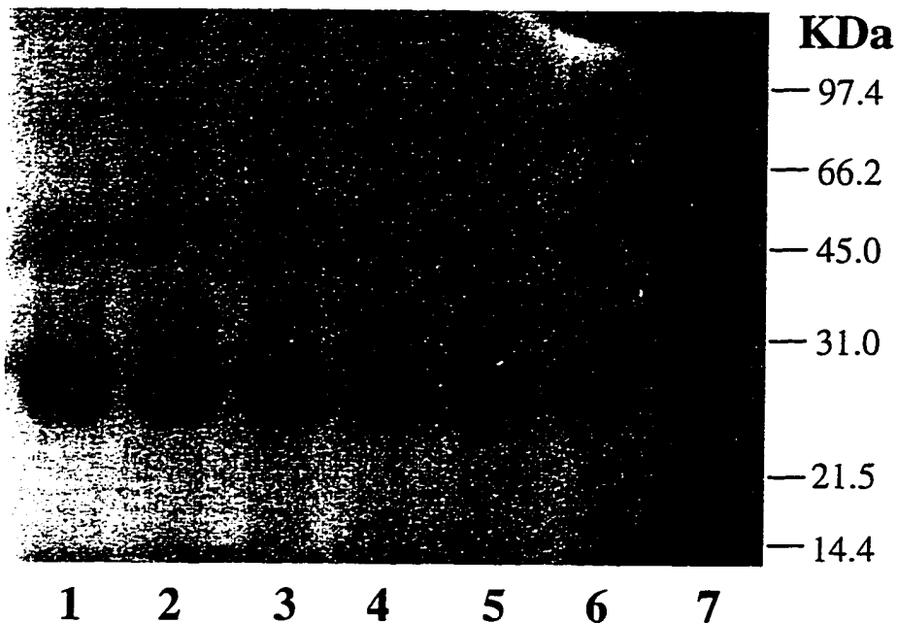


Figure 2

CHAPTER 6. GENERAL CONCLUSIONS

Proteinase inhibitors have been known as a part of plant defense system for almost 30 years. However, the mechanisms by which proteinase inhibitor genes are induced upon wounding still remain unknown. We have developed a molecular genetic approach to explore the mystery of the wound signaling pathway. Negative selection using the antimetabolite, 5FC, permits us to directly select mutants blocked in the wound-response.

A bacterial *codA* gene encoding for cytosine deaminase was expressed under the control of the wound-inducible *pin*2 promoter. The construct was transformed into *Arabidopsis*. The cytosine deaminase activity was detected in the transgenic plants (Tr349) induced by sucrose, ABA, methyl jasmonate and mechanical wounding. The induction of the *pin*2 promoter in the transgenic seedlings was also tested. Methyl jasmonate was found as the best candidate for the *pin*2 induction in transgenic Tr349 seedlings among all the wound signal chemicals tested. Induction was evident by the third day after seeds were plated on selective media and cytosine deaminase was highly active for at least 20 days. The effect of 5FC and 5FU was also tested on wild type and transgenic seedlings. Wild type seedlings had a dose-dependent sensitivity to 5FU. The seedlings were completely killed at a level of 100 μg/mL and 130 μg/mL 5FU for *Arabidopsis* and *N. tabacum*, respectively. 5FC at a concentration of 1 mg/mL had no effect on the seedlings of wild type of *Arabidopsis* and *N. tabacum* and *Arabidopsis* transgenic Tr349 seedlings without *pin*2 induction by methyl jasmonate. However, 5FC had a dose-dependent effect on the seedlings of transgenic Tr354 where *codA* gene was constitutively expressed and seedlings of transgenic Tr349 with the *pin*2 induction by methyl jasmonate. No seedlings survived at concentration of 500 μg/mL 5FC. Homozygous Tr349 seeds were mutagenized by EMS. M2 seeds were collected and plated out on selective media containing 50 μg/mL kanamycin + 25 μM methyl jasmonate + 500 μg/mL 5FC. About 110 seedlings from M2 seeds survived on selective media. M3 seeds were collected from these

M2 plants and rescreened on selective media to eliminate false positives. Based on cytosine deaminase assay and selection results, four M2 mutant lines that lost cytosine deaminase activity in M2 and M3 plants have been isolated. Genetic analysis will be needed to characterize these mutants.

In Chapter 3, the construct *pin*2-UMP^{Dd} for 5FOA negative selection was evaluated for its expression in tobacco at different levels. The construct was transformed into *N. tabacum* leaves. The presence of transgenes was confirmed by Southern blot analysis. The induction of the *pin*2 promoter by sucrose in transgenic Tr291 leaves resulted in an increase of mRNA levels of UMP synthase as measured by RNase protection assay and a significant increase in UMP synthase activity. These results indicated that the UMP synthase from *D. discoideum* functions in plants and its expression is regulated by *pin*2 promoter in Tr291 plants. The 2D-TLC analysis also showed that expression of exogenous UMP synthase in transgenic Tr291 plants altered nucleotide pool sizes.

We found that 5FC negative selection is better than 5FOA negative selection for mutant selection in our study. The advantage of using 5FC system is that plants lack cytosine deaminase activity. This overcame the problems that we had with 5FOA system.

Because of the long timeframes involved in the work with transgenic plants, several smaller projects were also conducted. Due to the success of these side projects, two of them are included in this thesis as separate chapters. Chapter 4 is the first report of isolation of UMP/CMP kinase from plants. This *Arabidopsis* UMP/CMP kinase showed 50% identity with other eukaryotic UMP kinases based on their deduced amino acid sequences. The cDNA was subcloned into a bacterial expression vector pGEX-4T-3. The UMP/CMP kinase was expressed as a fusion protein with glutathione-S-transferase (GST). The UMP/CMP kinase was purified to homogeneity by glutathione sepharose beads and a reverse phase HPLC following enterokinase digestion of fusion proteins. The identity of the purified UMP/CMP kinase was confirmed by N-terminal sequencing, amino acid composition analysis and MALDI

spectroscopy. Substrate specificity study showed that the enzyme prefers to use UMP (K_m = 153 μM) or CMP (K_m = 266 μM) as a phosphate acceptor and ATP (K_m = 29 μM) as a phosphate donor. Analytical ultracentrifugation analysis showed that the enzyme exists as a dimer. The optimal pH for the enzyme was 6.5. The kinetic mechanism was random Bi-Bi. Inhibition studies showed that a transition state analog AP_5A was a competitive inhibitor for both ATP and UMP.

Chapter 5 describes further studies on *Arabidopsis* UMP/CMP kinase in which the conserved amino acid residues were mutated in the phosphate-binding sequence (P-loop) for ATP. Like other eukaryotic UMP kinases, *Arabidopsis* UMP kinase also contains a glycine-rich sequence GGPGSGK at its N-terminus, which is homologous to the conserved sequence GXXGXGK found in many other ATP-binding proteins. To study the role of this conserved sequence in *Arabidopsis* UMP kinase, five conserved residues were mutated by PCR-based site-directed mutagenesis. Seven mutants were made: G21A, G22A, G24A, G26A, K27R, K27M and K27E. The G21A and G26A mutants were degraded after enterokinase digestion. All the rest mutants showed a decrease in k_{cat}/K_m value for ATP. A significant decrease in k_{cat}/K_m value for ATP was observed in the K27M and K27E mutants by 1000-fold and 2600-fold, respectively. The G22A and G24A mutants had a half of k_{cat} value of wild-type enzyme and 3.9-fold and 3.3-fold increase in K_m value, respectively. The K27R mutant had a decrease in k_{cat} value for ATP by 50% and almost no change in K_m value for ATP. The removal of the terminal positive charge of Lys27 in the K27M and K27E mutants resulted in 20% of k_{cat} value of wild-type enzyme and had a remarkable increase in K_m value for ATP by 228-fold and 537-fold, respectively. These results showed that the conserved residues in the P-loop, especially the terminal positive charge of Lys27 is important for ATP binding and enzyme catalysis.

APPENDIX A. VECTORS USED IN THIS WORK

Appendix Figure Legends

Plasmid	Purpose	Reference
pNE3	used as a template for PCR amplification of *E. coli codA* gene and TMV Ω sequence	Stougaard, 1993
pT7Blue(R)	used to subclone the Ω-*codA* PCR product	Novagen, Inc. Madison, WI
pRT24	a plasmid containing potato proteinase inhibitor II (*pin*2) promoter	Thornburg et al., 1987
pUC9	used to remove one SacI site from 3' end of the *pin*2-*codA* in pRT338	Promega Madison, WI
pRT346	used to remove the extra piece between *pin*2 and *codA* from pRT341	
pSP73	used to introduce a *Bgl*II site at 5' end of the *pin*2-*codA*	Promega Madison, WI
pRT38	a plasmid containing potato proteinase inhibitor II (*pin*2) terminator	Thornburg et al., 1987
pRT339	a *pin*2 terminator-containing plasmid with an introduced *Bgl*II site	
pGA482	a plant transformation binary vector	An, 1987
pRT349	a plant transformation binary vector containing *pin*2 promoter, *codA* gene and *pin*2 terminator	
pNit36.3	used as a template for PCR amplification of *Arabidopsis thaliana* nitrilase 1 promoter	Zhou et al., 1995
pRT354	a plant transformation binary vector containing nitrilase 1 promoter, *codA* gene and *pin*2 terminator	
pRT291	a plant transformation binary vector containing *pin*2 promoter, $UMPS^{Du}$ gene and *pin*2 terminator	Shi, 1991
pRT292	a plant transformation binary vector containing CaMV 35S promoter, $UMPS^{Du}$ gene and *pin*2 terminator	Shi, 1991
pAt-ura6	a pFL61 yeast expression vector containing *Arabidopsis thaliana* UMP/CMP kinase cDNA	Minet et al., 1992; Zhou et al., submitted
pGEX-4T-3	a bacterial expression vector containing a glutathione-S-transferase (GST) as fusion partner	Pharmacia Biotech, Piscataway, NJ
pRT380	a bacterial expression vector containing GST gene and *Arabidopsis thaliana* UMP/CMP kinase cDNA	

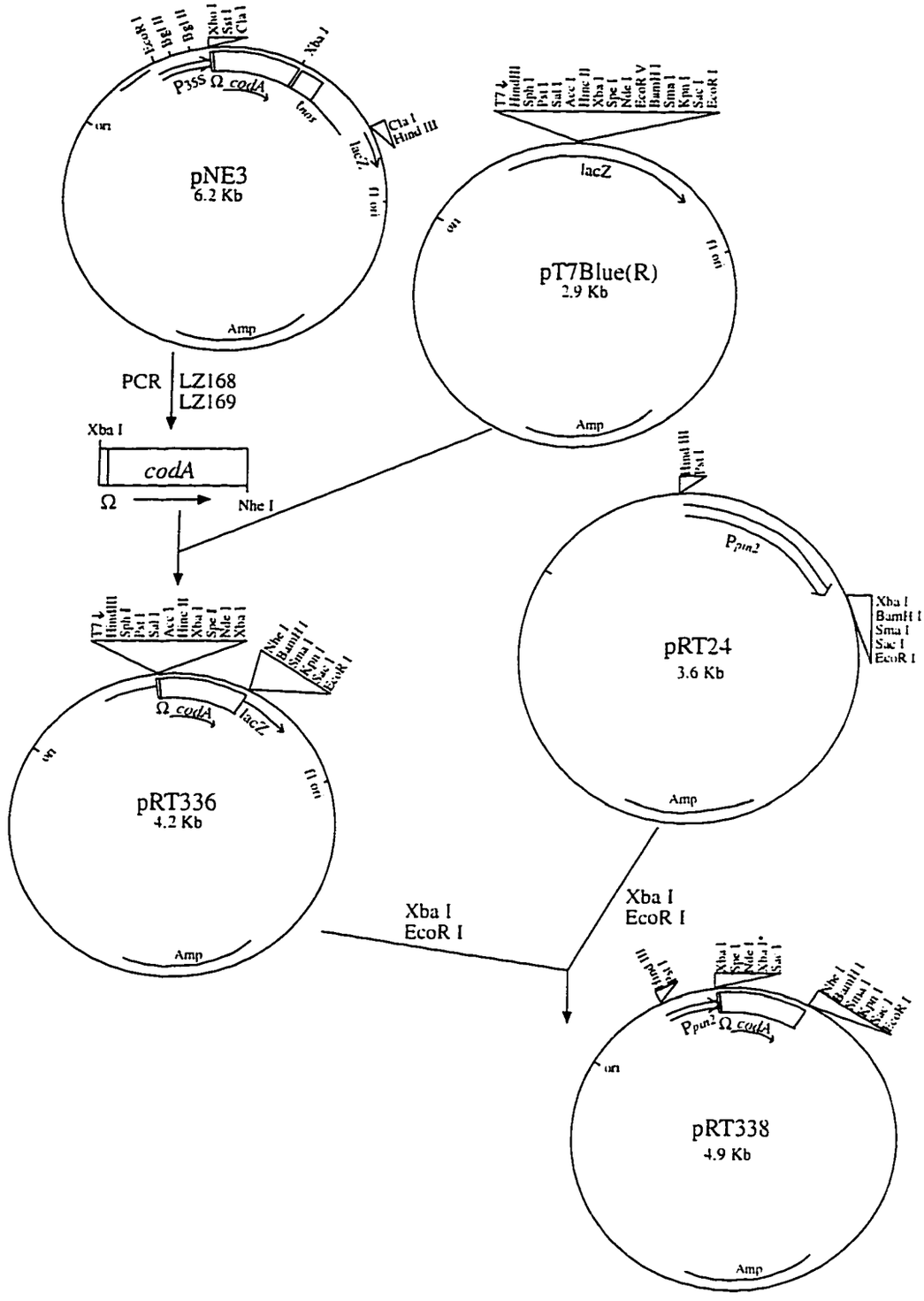


Figure A1 Construction of the vector pRT349

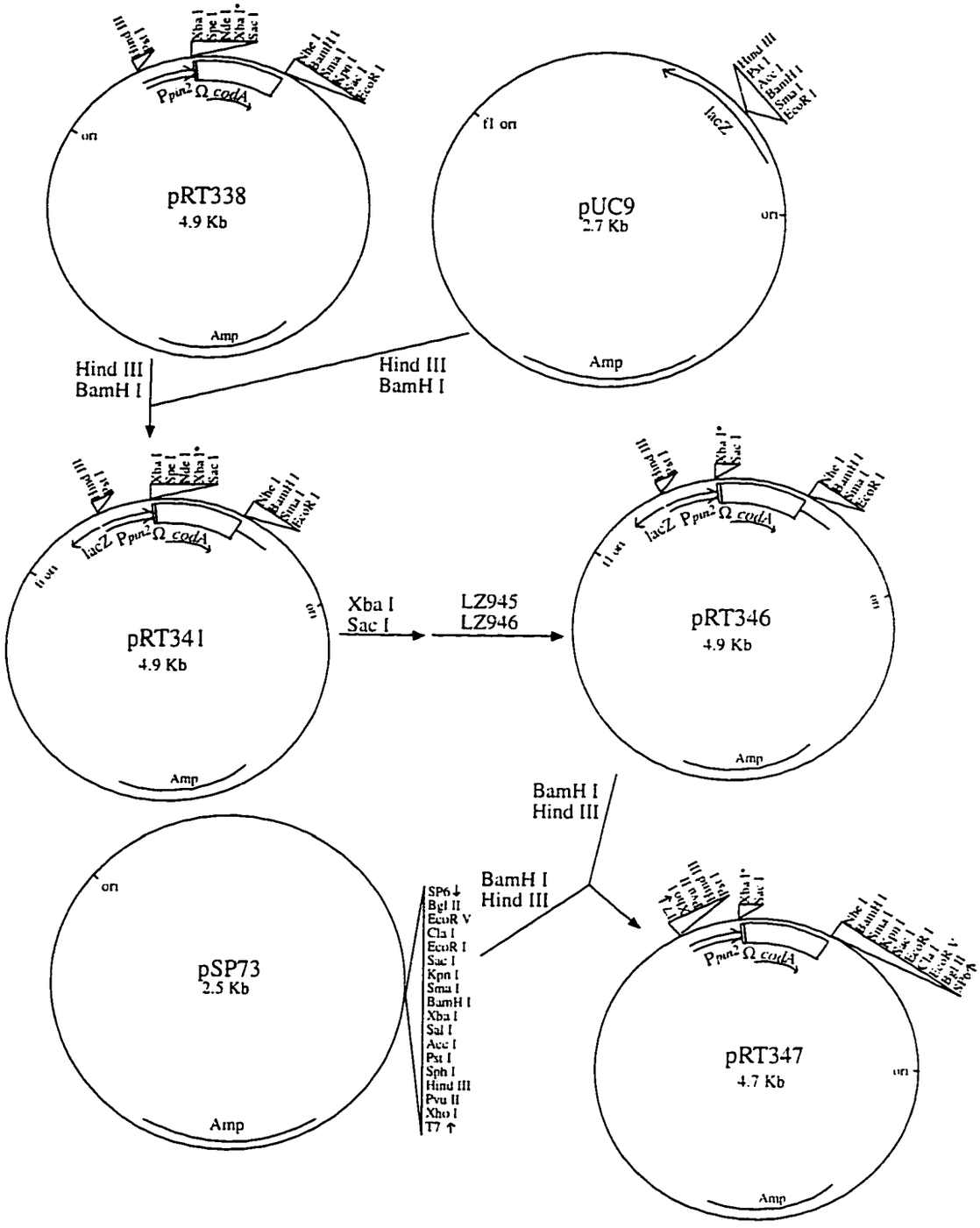


Figure A1 (continued)

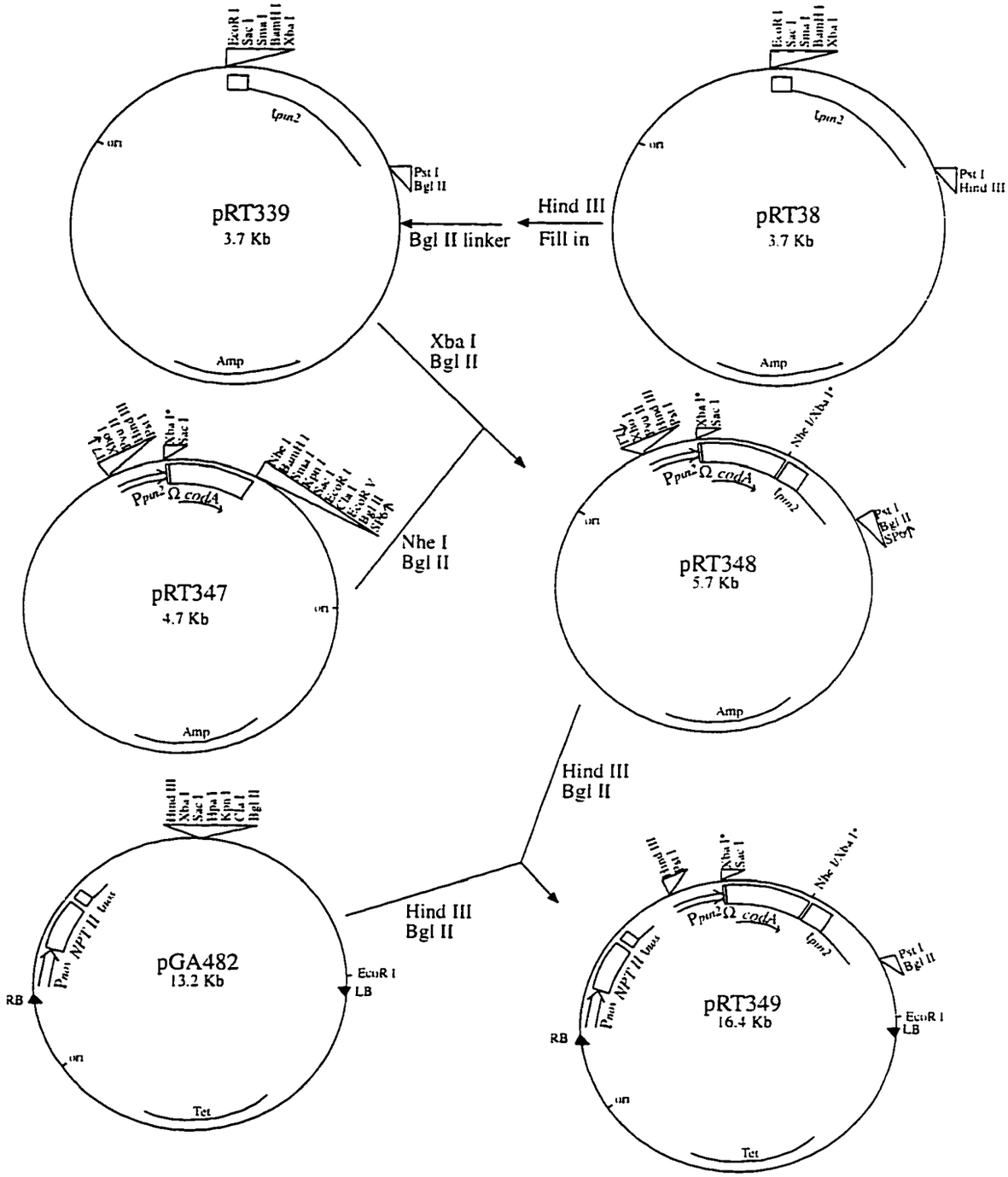


Figure A1 (continued)

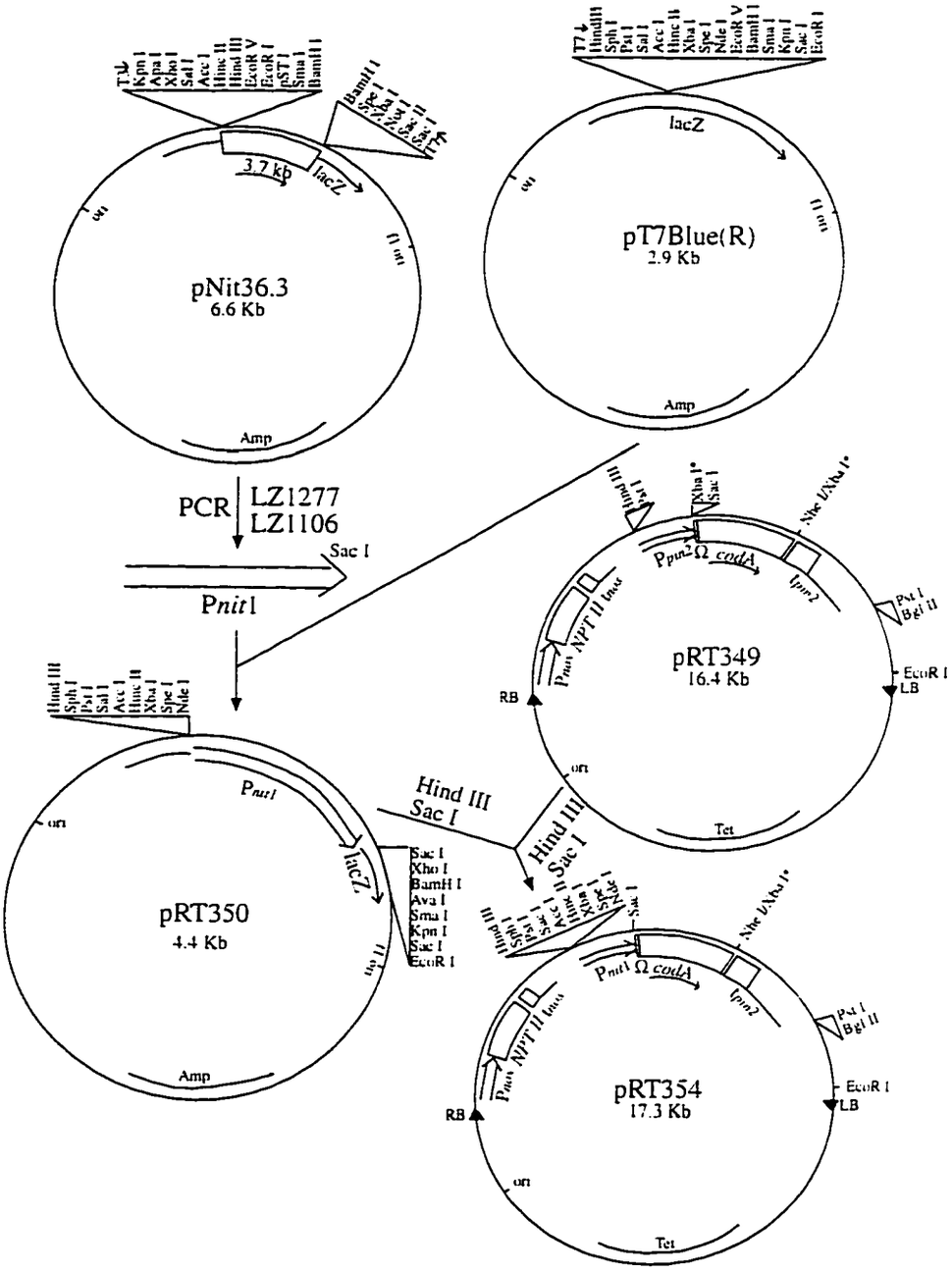


Figure A2 Construction of the vector pRT354

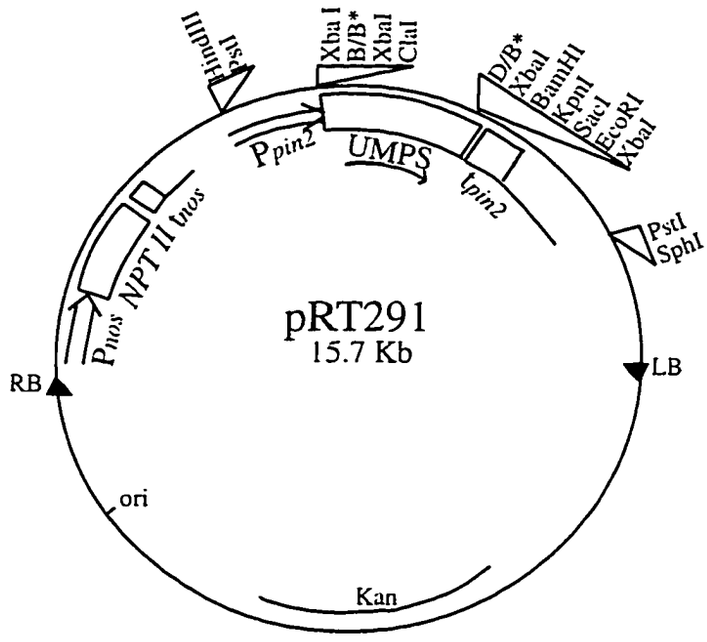


Figure A3 The vector pRT291

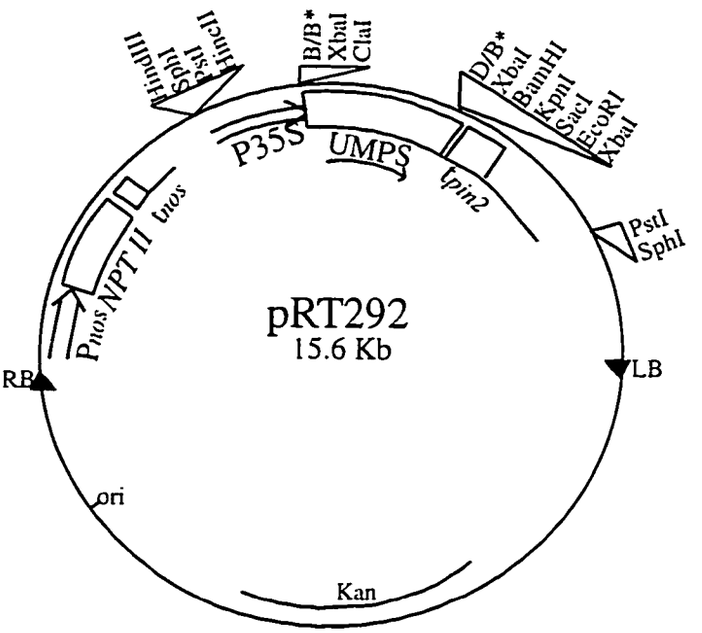


Figure A4 The vector pRT292

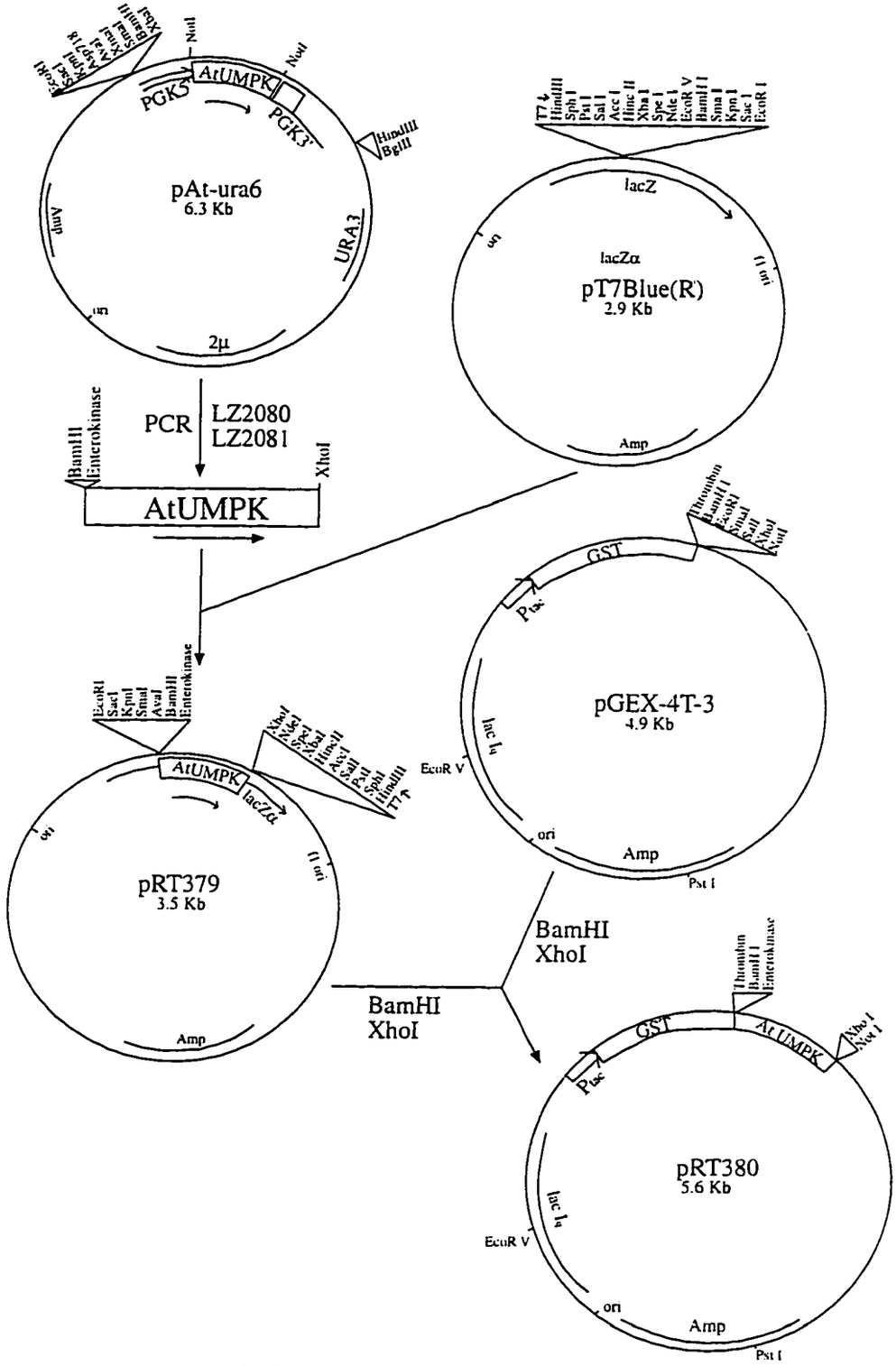


Figure A5 Construction of the vector pRT380

APPENDIX B. OLIGONUCLEOTIDES USED IN THIS WORK

Oligonucleotide	Sequence	Number of nucleotides	Use
LZ168	5'-GGGGCTAGCTCAACGTTTGTAATCG-3'	25	PCR amplify *codA* gene
LZ169	5'-GGGTCTAGATCGAGTATAAGAGC-3'	23	PCR amplify *codA* gene
LZ697	5'-CAGATGAATCTGCCACAG-3'	18	sequence the junction between *codA* gene and its 5' upstream
T7-1	5'-AATACGACTCACTATAG-3'	17	sequence an insert in pT7Blue(R) or pBluescript II SK (+/-)
Universal (U)	5'-TGTAAAACGACGGCCAGT-3'	18	sequence an insert in pT7Blue(R)
LZ945	5'-CTAGATCGAGTATAAGAGCT-3'	20	replace a removed fragment of TMV Ω sequence when the extra piece in pRT341 was removed
LZ946	5'-CTTATACTCGAT-3'	12	replace a removed fragment of TMV Ω sequence when the extra piece in pRT341 was removed
*Bgl*II linker	5'-GGAAGATCTTCC-3'	12	introduce a *Bgl*II site into pRT38
LZ1277	5'-TTATTATGAATCTGGCTTTG-3'	20	PCR amplify nitrilase 1 promoter
LZ1106	5'-CTCGAGCTCTTTGTTTTTTACTTTGGT-3'	27	PCR amplify nitrilase 1 promoter
LZ2080	5'-GCGGATCCGATGACGATGACAAGATGGGATCTGTTGATGCTGCT-3'	44	PCR amplify *Arabidopsis thaliana* UMP/CMP kinase cDNA
LZ2081	5'-CGGCTCGAGCTACTAGGCTTCAACCTTCTCAGC-3'	33	PCR amplify *Arabidopsis thaliana* UMP/CMP kinase cDNA
VI0236	5'-CCCACTTCCATTAGCA-3'	16	sequence 5' end of *Arabidopsis thaliana* UMP/CMP kinase cDNA
VI0237	5'-GCTGTCTTCGAGGAGGT-3'	17	sequence 3' end of *Arabidopsis thaliana* UMP/CMP kinase cDNA
T3	5'-ATTAACCCTCACTAAAG-3'	17	sequence *Arabidopsis thaliana* UMP/CMP cDNA mutant in pBluescript II SK (+/-)
LZ2436 (pGEX-1)	5'-GGCATCCGCTTACAGACAAGCT-3'	22	sequence *Arabidopsis thaliana* UMP/CMP cDNA mutant in pGEX-4T-3
LZ2453 (pGEX-2)	5'-CGTATTGAAGCTATCCCACAA-3'	21	sequence *Arabidopsis thaliana* UMP/CMP cDNA mutant in pGEX-4T-3

ACKNOWLEDGMENTS

I would like to take this opportunity to thank all the people who supported, encouraged and helped me during my Ph.D. study.

I would like to give my sincere thanks to my major professor Dr. Robert W. Thornburg for his guidance, support and patience for the last five years, and particularly for giving me the freedom in designing and conducting experiments. Dr. Thornburg has always been helpful and understandable and I was impressed as well as encouraged not only by his charming personality, but also by his dedication and enthusiasm toward research.

I would like to thank other committee members: Dr. Basil Nikolau, Dr. Dan Voytas, Dr. Jan Buss and Dr. Randy Shoemaker for their time and helpful suggestions on my research projects.

Thanks also go to my parents for their understanding, encouragement and love.